THE UREACLASTIC MICRO-ORGANISMS OF SOILS

BY

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PREFATORY NOTE

This group of papers describes a study of the microbiology of urea. The chief objects of the work were the identification and classification of the organisms responsible for decomposing urea in soils, a study of the factors controlling their vital activities, and an investigation of their action in soils of varied character. Although the papers were published in four different journals they form a continuous series. The first six deal with the pure bacteriology of the problem, while the last two papers are concerned with quantitative aspects of the decomposition in soils.

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AN INVESTIGATION OF THE BACILLUS PASTEURI GROUP

I. DESCRIPTION OF STRAINS ISOLATED FROM SOILS AND MANURES

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In 1889, Miquel briefly described *Urobacillus Pasteurii*, a sporing bacillus found in rivers and sewage which brought about an exceptionally active decomposition of urea. Beijerinck (1901) gave a fuller account of an organism isolated from soils and dust which he considered to be *Urobac. Pasteurii*; but the first detailed description of the bacillus was published by Löhnis and Kuntze (1908) who referred to the organism as *Bacillus Pasteuri* (Miquel) Migula. A number of other urea-decomposing bacteria which have been described are difficult to distinguish from *B. Pasteuri* and undoubtedly belong to the same group. In this category may be mentioned *Urobac. Duclauxii* and *Urobac. Maddoxii* (see Miquel, 1904), *Urobac. Freudenreichii* Miquel which was fully described by Löhnis (1905) as *Bacillus Freudenreichii* (Miquel) Migula, *Urobac. leubei* Beijerinck, *Bacillus probatus*, A. Meyer et Viehoever, *Urobac. psychrocartericus* Rubentschik and *Urobac. hesmogenes* Rubentschik. The classification of these bacteria is still one of the least satisfactory parts of systematic bacteriology. The uncertainties in classification appear to have arisen partly because certain species were imperfectly described and partly because most workers who made detailed descriptions of their organisms worked with comparatively few strains. It is noteworthy that Geilinger (1917), who investigated a larger number of strains than any of his predecessors, experienced more difficulty than others in classifying the organisms. Since the work of Beijerinck (1901) and Löhnis

and Kuntze (1908), the most clearly characterised organism of the group has generally been considered to be *B. Pasteuri*; but the distinctions between this type and other sporulating urea decomposers have never been precisely defined.

A consideration of the literature dealing with this group of organisms suggested that their classification could only be clarified by a comparative study of a large number of strains supplemented by an investigation of their variability under the conditions of artificial culture. Work was undertaken on these lines and the results of a study of 94 freshly-isolated strains are presented in this paper. The variability of the pure cultures was explored partly by the separation of variants by plating and partly by the reëxamination of stock cultures which were transferred every two months. Fifty-three strains were cultivated on urea agar for four years: a proportion of them were examined at intervals and all were investigated again at the end of the period.

SPECIAL MEDIA AND METHODS

The media found to be most suitable for studying pure cultures are bouillon, agar and gelatin in which 2 per cent urea is dissolved immediately before sterilisation. They will be referred to as urea bouillon, urea agar, etc. These media contain 1 per cent peptone and 1 per cent meat extract. Lower concentrations are less satisfactory, probably on account of smaller buffering capacity in the neighbourhood of pH 9. In order to obtain media free from precipitates produced by the breakdown of urea during sterilisation, the bouillon is prepared by adjusting the reaction to approximately pH 8.5 by adding NaOH and autoclaving: after filtration the reaction is brought back to approximately pH 7 by adding HCl and the remaining ingredients are dissolved. Intermittent steaming is the most suitable method of sterilisation. Urea readily decomposes in the autoclave and although this may not destroy the growth-promoting properties of the media, it leads to excessive precipitation. Media containing urea sterilised by filtration do not encourage growth to the same degree as those in which the urea is sterilised by heat. A freshly prepared medium is superior to one which has been stored for some

time. Agar containing 0.25 to 1.0 per cent urea is satisfactory for maintaining stock cultures, especially those of the less active strains, but is inferior to 2 per cent urea agar as a plating medium.

With the object of securing a representative series of organisms, pure cultures were isolated from a variety of sources by different methods. In every case primary cultures were prepared in media containing 5 or 10 per cent urea in order to utilise the special tolerance of ureaclastic organisms to high concentrations of this compound and to the ammonium carbonate which they produce from it. Three main methods were used.

1. Enrichment cultures were prepared by inoculating urea-containing solutions and incubating at 22°, 30° or 37°C. The media used were bouillon and soil extract (1 kgm. garden soil and 1 l. tap water autoclaved to 22 pounds, filtered, the filtrate made up to 1 l. and 0.05 per cent K_2HPO_4 added), the latter with or without the addition of 1 per cent glucose or glycerol. Usually a 5 gram quantity of soil or manure was added to 50 ml. of medium in an Erlenmeyer flask. In a few cases deep tubes were used with the object of restricting aeration. The course of the decomposition was followed by periodically titrating 1 ml. quantities of the cultures against standard H_2SO_4 and isolations were made at different stages. All the different forms of enrichment culture just described appear to lead to the same result: they allow the active urea-decomposing bacteria in the sample to grow more rapidly and outnumber inactive organisms. Even when isolations were made early in the development of the cultures, the majority of the organisms secured proved to be active types. Variations in the medium, in the temperature of incubation or in aeration did not appear to influence the types of bacteria which developed except that it was necessary to add about 10 per cent urea to bouillon and about 5 per cent urea to soil extract in order to prevent completely, during the early stages of incubation, the growth of organisms which do not decompose urea.

2. In order to isolate organisms which might be incapable of multiplying rapidly in enrichment cultures and yet occur in large numbers in soils, the dilution method was used. Dilutions of soils were prepared and tubes of 10 per cent urea bouillon or

5 per cent urea soil extract were inoculated from a series of dilutions. In view of Millard's (1912) observations on the use of the dilution method for soil bacteria, about 0.5 gram of soil was sterilised in each tube by autoclaving prior to adding the media and sterilising by intermittent steaming. The inclusion of soil in the media had, apart from its possible nutritive properties, the advantage that if any appreciable decomposition of urea took place the culture became brown as a result of the solution of humus by ammonia. The organisms isolated from the highest dilutions containing urea decomposers were invariably among the least active types.

3. Direct plating of soil or soil dilutions on agar was used in a search for organisms for which methods 1 and 2 are unsuited. Flasks containing 100 ml. of nutrient agar (2 per cent agar, 1 per cent peptone and 1 per cent meat extract) were prepared and immediately before use the medium was melted, 5 or 10 per cent crystalline urea was added, and, after steaming for 10 minutes, the medium was cooled and used for plating. The soil or soil dilution was either mixed with the melted agar or spread on the surface of the medium after hardening. Colonies of urea-decomposing bacteria generally produced precipitates of microscopic crystals in the agar surrounding them as a result of the intense alkalinity developed; but colonies which appeared late or tended to spread did not always show this appearance. The agar containing 10 per cent urea suppresses practically all organisms which do not decompose urea but it is also more inhibitive to ureaclastic bacteria than the less selective 5 per cent urea agar. The cultures isolated by direct plating of soils were invariably types possessing feeble urease activity.

Most of the strains were isolated on 2 per cent urea agar. Beijerinck's (1901) urea yeast-extract gelatin, which is also a suitable medium, was used for a few isolations. The gelatin possesses some advantage in showing crystal formation around the colonies of urea-decomposing organisms. Several other solid media were tried but were found to be unsatisfactory. Colonies appearing on primary plates were replated at least once before cultures were assumed to be pure. The urease activity of pure

cultures was tested in bouillon containing 2 per cent urea, the course of the decomposition being followed by titrating 1 ml. quantities of the cultures against standard acid. Tests for residual urea were made, when necessary, with soybean urease.

The majority of the bacteria which were isolated belong to the *Bacillus Pasteuri* group and they alone will be described in this paper. Isolations were made from 38 samples taken from 6 types of soil, 32 strains being obtained from enrichment cultures, 19 from liquid media inoculated with high dilutions and 37 by direct plating. The remaining 6 strains were isolated from enrichment cultures inoculated with farmyard manure.

DESCRIPTION OF PURE CULTURES

It may be indicated here that the possibility of classifying this group of bacteria in clearly defined species is not promising and the only form of description which appears to be satisfactory is one which surveys the group as a whole. Accordingly, the series of recently-isolated strains will be described as a single entity, but reference will be made to any observations which might be of importance for purposes of classification.

Microscopic characteristics

Morphology. The organisms are rod-shaped with rounded or slightly pointed ends. Chain formation may be observed in preparations of living cells, but seldom in stained films. There are two main morphological types. In one (plate 1, figs. 1 and 2) the stained cells are 0.5 to 1μ thick and variable in length: spherical or slightly ovoid spores, which are usually greater in diameter than the sporangia, are formed chiefly in a terminal or sub-terminal position. In two strains (fig. 3) the spores are exceptionally large and measure 1.5μ or more in diameter. In the second main type (figs. 4 and 5) the cells are comparatively short and frequently almost coccoid: they are seldom more than 3μ in length and they vary from 0.6 to 1.5μ in thickness. In these strains the spores are ovoid to elliptical: they are formed in a central or excentric position and they may occupy most of the cell interior.

The shape and size of the vegetative cells are moderately constant strain characteristics so long as stock cultures are maintained in the usual way, but stable modifications in morphology may be produced when variants are isolated by plating. Organisms which grow almost entirely as long undivided filaments (fig. 6) may be obtained in this way. The shape of spore appears to be a stable character but its value as a basis of classification is reduced by two circumstances. Firstly, there is no definite division between the two main types described above for every possible intermediate form has been encountered and, secondly, among organisms of this group the capacity to form spores usually becomes much reduced and may be completely lost during artificial cultivation. Spore formation occurs most readily on solid media comparatively poor in nutrients and approaching in their reaction the limiting acidity tolerated by the organisms. Media suitable for eliciting the production of spores are potato and ordinary agar prepared from a dilute bouillon, provided growth can be induced on these substrates. The heat resistance of the spores is variable. A few cultures when tested immediately after isolation contained spores which survived exposure at 98° to 100°C. for fifteen minutes. Others were destroyed at the same temperature almost immediately. In general, the more elongate the spores, the greater is their resistance to heat.

Cells of irregular shape are seldom formed. A few strains occasionally produce spherical to spindle-shaped forms 2 to 5μ in diameter. These are probably the cells which Miquel (1905) considered to be typical of *Urobac. Maddoxii*. Certain strains or strain variants—chiefly those which grow as long rods—exhibit a tendency to form small non-motile coccoid bodies (plate 2, fig. 7). These bodies develop from minute buds and correspond in their characteristics to one of the types of bacterial gonidia. Strains which no longer form heat-resistant spores may produce spherical bodies (fig. 8) which are usually smaller than spores and are unstainable in their mature condition except by spore-staining methods. These structures are destroyed immediately at 80°C. They generally originate as Gram-positive buds and they correspond therefore to "regenerative bodies" as defined

by Löhnis (1921). There can be little doubt, however, that in this group of bacteria they represent a degraded form of spore.

Motility. With one exception—an organism which has not been observed to form spores—all the strains were motile when examined soon after isolation. The arrangement of the flagella is peritrichous, but in some strains few of the cells appear to be furnished with more than 2 flagella. Several of the variants isolated from particular strains by plating proved to be non-motile. These were chiefly long slender rods and the filamentous types shown in figure 6, but two of them were comparatively short rods.

Staining. The rods stain readily with aqueous fuchsin and seldom show any differentiation of their protoplasm. The reaction to Gram's method of staining is variable. The thicker rods are generally Gram-positive but the thinner organisms are easily decolorised and may be frankly Gram-negative. With modifications of Gram's method in which acetone is used most strains are decolorised instantly.

Conditions of growth

The optimum temperature varies among different strains from about 25°C. to about 37°C. Strains showing the highest optimum have a maximum temperature of about 42°C. Those with the lowest optimum do not grow at 30°C. or at higher temperatures. The minimum temperature of the latter types is about 0°C. Among the strains which have temperature maxima under 30°C. there are no examples of organisms forming elongate spores or capable of producing a rapid decomposition of urea.

The organisms are aerobic but the oxygen requirement, as shown by stab and shake cultures in agar, varies to some extent among different strains.

The results of experimental work on nutrient requirements and on the effect of reaction will be presented in a subsequent paper.

Most strains, including the non-sporing types, remain alive on urea agar for several months. The viability, especially of types which produce a thin growth, is considerably prolonged if the agar contains a low concentration of urea (0.25 to 0.5 per cent)

and if drying is prevented. If growth is secured on ordinary agar, the organisms remain alive for several years.

Cultural characteristics

Urea-containing media are generally referred to in the following account of cultural characters. Strains which decompose urea actively are almost incapable of developing in ordinary neutral media, while only a few of the least active strains grow readily in these substrates. In the case of strains capable of growing in ordinary media, the cultural characteristics are not appreciably modified by the presence of urea.

Colonies on urea agar. The following are the chief types of surface colony produced by different strains or by variants of the same strain:

Type 1. Small (up to 1 mm.), round, translucent; bluish by transmitted light. Microscopically finely granular; edge entire. This type is illustrated on plate 2, figure 9.

Type 2. Larger (up to 3 to 4 mm.), round, grey and glistening; becoming thick, opaque, and by transmitted light yellowish-grey. Microscopically finely granular without other features, later opaque; edge entire. Illustrated in figure 10.

Type 3. At first translucent and granular by transmitted light; later opaque and yellowish in the centre, and translucent and granular in the marginal zone; edge ragged; up to 1 cm. in diameter. Microscopic structure resembles folded hair. Illustrated in figure 11.

Type 4. A series of thin colonies varying from the type illustrated in figure 12, which has a comparatively smooth surface, to much thinner and more irregular forms which show a folded hair structure microscopically.

The different types of colony appear to be determined mainly by the morphology of the organisms. Colonies of type 1 are generally produced by the smaller rods, those of type 2 by short thick bacilli, while those of types 3 and 4 are formed by long rods and filaments. The form of colony, within the range of types described, can be of only limited value from the systematic standpoint. It is entirely unrelated to the biochemical proper-

ties of strains, it exhibits every grade in type between the extremes, and it is not specially stable. After the isolation of a pure culture, the form of colony tends to change in the direction of the "rough" types 3 and 4, a process which may be greatly accelerated by making serial transfers in urea bouillon and isolating distinctive variants.

Colonies on urea gelatin. Colonies formed on gelatin by different strains form a series of types similar to those on agar. The distinctions between types are generally more clearly marked in gelatin colonies, but a classification of the various forms is complicated by the variable rate of liquefaction of the medium and the modifications of colony structure thus produced. The surface colonies may either develop into circular liquefactions or form hollows or they may show no evidence of acting on the gelatin. Microscopically, they may become ciliated, hairy, or diffuse, or they may produce flame-like outgrowths, depending on the rate of liquefaction. Deep colonies are usually dense and round, occasionally irregular. At a later stage they may produce microscopic hair-like or twisted outgrowths or lobes. The colonies of some strains may resemble those of Rubentschik's (1926) *Urobac. hesmogenes.*

Urea agar slope. Conforming to the type of colony, the growth on slopes varies in different strains from a thin transparent layer to one which becomes thick and yellowish-grey by transmitted light. The growth has a glistening surface and may be smooth or may have a granular appearance. There is no tendency to rapid spreading.

Urea gelatin stab. The stab growth is at first thread-like, but most strains later form outgrowths from the upper part. The production of outgrowths is not a constant characteristic of particular strains for it depends in part on the composition of the medium. The surface growth is similar to colonies in Petri dishes. The rate of gelatin liquefaction varies greatly among different strains but is never rapid. The most active strains produce in seven days a small saucer-shaped liquefaction which later becomes cup-shaped and then cylindrical. Many strains form an empty cup-shaped or napiform hollow, from which the

liquid evaporates almost as rapidly as it is formed, unless the tubes are closed. The least active strains may produce only slight softening of the surface after several months in capped tubes and may show no action on some samples of the medium within one year. Two strains have failed entirely to produce any action on gelatin. The rate of liquefying gelatin does not correlate with any other characteristic of the organisms.

Urea bouillon. Most strains produce a turbidity of varying intensity and later a slimy sediment. A few form a soft pellicle which readily breaks up and sinks in flocculent masses.

Potato. Usually no growth appears. Strains which grow fairly readily on ordinary agar may produce some grey to brown growth if the medium is heavily inoculated and especially if much ammonia is being formed in the incubator.

Biochemical characteristics

Milk. Usually no change takes place. The strains which grow most readily on ordinary media may, if heavily inoculated into milk, slowly produce an alkaline reaction. This change may be followed by a slight discoloration after six weeks or more at the optimum temperature of the organism, and a slow peptonisation may occur during subsequent months. Most strains grow fairly readily in milk to which 2 per cent urea has been added after sterilisation. In this medium the preliminary clearing, brought about by the formation of ammonia, is followed by a slow digestion of proteins and the milk is converted into a semi-clear, brownish liquid after several weeks. The reduction of litmus in milk is slow and variable.

Nitrates. In urea bouillon or semi-solid agar some strains reduce nitrates to nitrites and others do not. Nitrites are not reduced. Nitrate-reducing ability is not associated with any other character which has been found to distinguish different strains.

Indol is not formed as a rule but certain strains produce traces in tryptophane-containing media.

Diastatic action and the formation of *hydrogen sulphide* are not demonstrable by the usual methods.

Glucose bouillon is rendered alkaline by most strains capable of growing in the medium. Parallel cultures in ordinary bouillon generally become alkaline more rapidly, thus indicating that some acid is formed from the sugar. A few strains produce a slight acidity (pH 6.5 to 7).

Urea-decomposing activity. There is a wide variation in the rate of action on urea. The most active strains completely decompose 2 per cent urea in bouillon within twenty-four hours at their optimum temperature, even when the medium is only lightly inoculated. The least active strains are incapable of ammonifying the same concentration of urea within ten days; and between the two extremes there are strains which exhibit all gradations of urease activity. There is no connection between rapidity of growth in the medium and urea-decomposing ability. Both vigorously- and weakly-growing strains may produce either an active or a slow decomposition. This observation suggests that Viehoever's (1913) tedious method of characterising these organisms by finding the relation of multiplication to urea hydrolysis is unsatisfactory. Several observers have reported on the loss of urease activity in different bacteria, but in the Pasteuri group the ureaclastic property appears to be a relatively stable characteristic. Of 53 strains which were cultivated on agar for four years, only one—an organism which at the end of the period was no longer motile and tended to grow in long filaments—lost the ability to produce a titratable alkalinity in urea bouillon. With this exception, the greatest reduction in activity observed was a lengthening of the time required for the complete decomposition of 2 per cent urea in bouillon, from under twenty-four hours to nearly four days. Some irregularity occurs in these tests for the results are influenced by the length of the lag period, which chiefly depends on the amount of inoculum and on the freshness of the medium.

Ureaclastic activity appears to be one of the few possible characters upon which to base a classification of the group. It possesses the disadvantage of arranging strains in an unbroken series, but it is correlated with media requirements and, to some extent, with morphology. The greater the urease activity of a strain,

the more unsuitable are the ordinary bacteriological media for its cultivation; and none of the strains which form elliptical spores bring about a rapid decomposition of urea.

CLASSIFICATION OF RECENTLY-ISOLATED STRAINS

Careful consideration has been given to the problem of classifying the series of strains described here and of identifying the different forms with previously-described types. A number of the organisms appear to correspond completely with *B. Pasteuri* as described by Löhnis and Kuntze (1908) and others with *B. Freudenreichii* as characterised by Löhnis (1905). *Urobac leubei* Beijerinck (1901) and certain of Rubentschik's (1926) organisms also appear to be represented. It is impossible, however, to allot every strain to a named species. The earlier investigators, working at different dates, did not employ the same criteria for characterising their species, and in certain cases it is impossible to decide whether they were describing the same or different types. In addition, the series of strains examined in this work appears to embrace a wider range of types than that represented by published descriptions. It is obvious that the classification of the group requires revision.

The observations cited in the description indicate that the recently-isolated strains cannot be grouped easily in well-defined types. Clear divisions are lacking between the extreme morphological and cultural forms, while biochemical characters are associated in almost every possible combination. With the object of providing information which would be useful in classifying the organisms the variability of the strains under laboratory conditions was investigated. The chief changes observed in stock cultures maintained on agar for four years were a decrease in spore-forming ability, some reduction in urease activity, and a change, in the case of certain strains, in the form of colony to a "rougher" type. A few representative strains were employed in attempts to stimulate variation by making serial transfers in urea bouillon and plating the cultures on urea agar. Each of the strains submitted to this procedure produced more than one type of colony, there being a general tendency to the production

of "rougher" colonies. Cultures derived from distinctive colonies were subjected to further study, and a proportion of them appeared to be stable variants. This work showed that the differences between strains in respect of the morphology of vegetative cells and the characters of growths on solid media are not constant and are therefore unreliable as an aid to classification. One strain produced the entire series of colony types illustrated on plate 2 and also intermediate forms. The shape of spore was never observed to change, but most stabilised variants lost the ability to form spores. The physiological properties appeared to be the most constant characters of the organisms. Where differences in physiology among the variants of a strain were recognised, they were chiefly of a quantitative nature. The experiments therefore shed no light on the irregular distribution of biochemical characteristics among different strains.

It is impossible to avoid the conclusion that a natural subdivision of this group of bacteria cannot be made and any classification must be arbitrary. Nevertheless, it seems to be desirable to suggest some form of subdivision which will permit of reference in a general way to the salient types within the group. The most suitable proposal appears to be the separation of organisms which decompose urea actively, and are incapable of growing in ordinary neutral media, from the remaining types. The former produce spherical or slightly ovoid spores. The latter might be divided into those which form approximately spherical spores and those which produce ovoid to elliptical spores. The problem of naming these types cannot be dealt with here, but a future paper of this series will contain a report on a number of cultures which were preserved by previous investigators, and the evidence presented will be used in making proposals on nomenclature.

SUMMARY

A description is given of 94 strains of urea-decomposing bacteria which may be placed in the *Bacillus Pasteuri* group. The organisms were isolated from a variety of soils and from manures (1) by means of enrichment cultures in media containing 5 or 10

per cent urea, (2) by inoculating urea-containing liquid media with high dilutions of soils, and (3) by direct plating of soils on agar containing 5 or 10 per cent urea. Method (1) permits of the isolation of the most active urea-decomposing bacteria in a sample, while methods (2) and (3) yield cultures of the most numerous and generally the least active organisms. The group consist of a series of strains among which there appear to be no natural divisions. Strains which decompose urea actively are almost incapable of developing in ordinary neutral media but a few of the least active organisms grow readily in these substrates. Individual strains exhibit variations in the morphology of vegetative cells and spores, the optimum temperature (from about 25° to about 37°C.), the form of colony, and the action on gelatin and nitrates. The variability of pure cultures was explored partly by the separation of variants by plating and partly by re-examining stock cultures at intervals. The study of colony variants proved that differences between strains in respect of cell morphology and the characteristics of growths on solid media are not constant, but the physiological properties of variants are in general identical with those of their parent strain.

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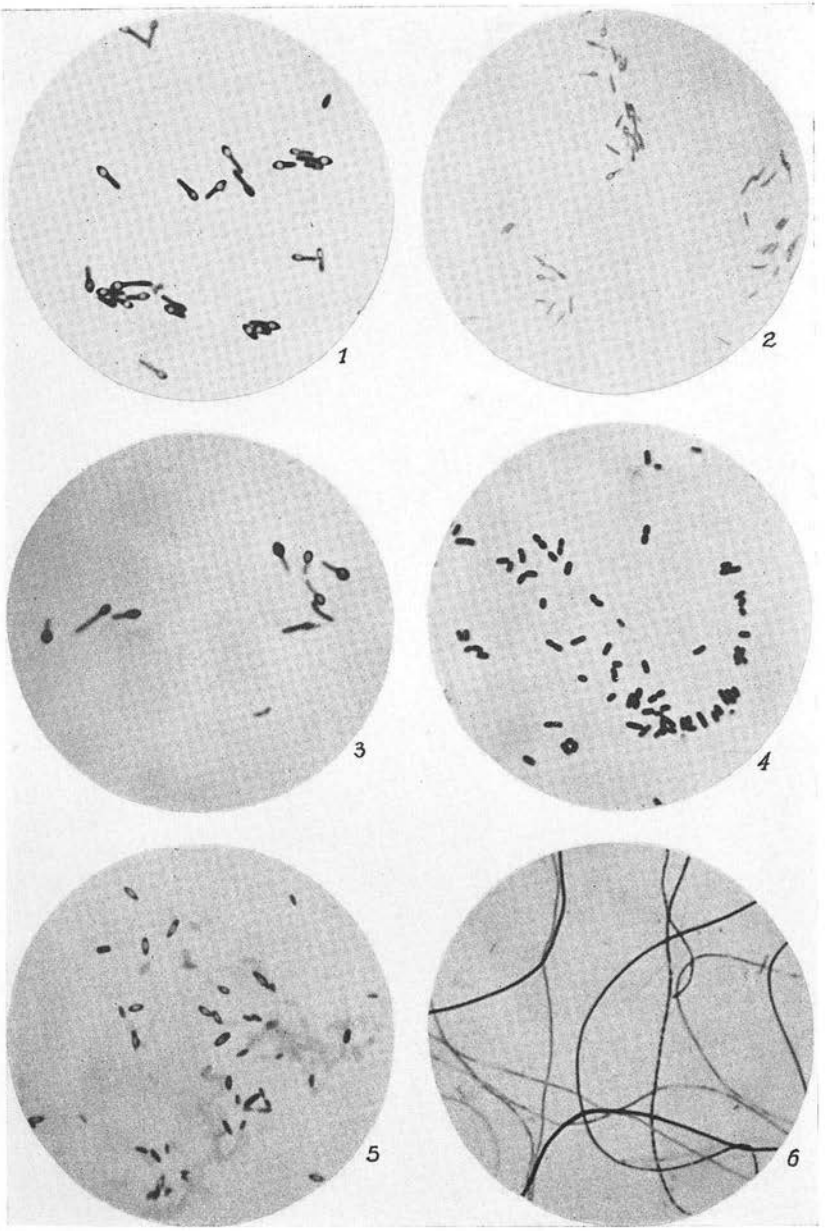
PLATE 1

Preparations from cultures on agar containing urea or ammonia, incubated at 30°C. for one to three days. Stained with cold aqueous fuchsin. Magnification ×1000.

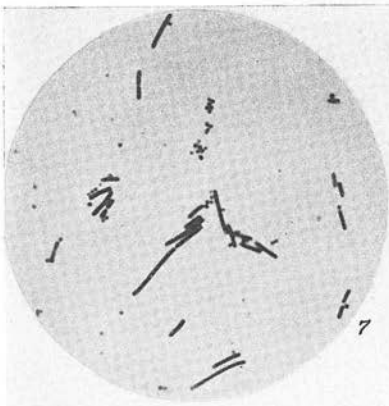


(Gibson: Investigation of Bacillus Pasteuri group)

PLATE 2

FIGS. 7 AND 8. Preparations from cultures on urea agar incubated at 30°C. for two days (fig. 7) and three days (fig. 8). Stained with cold aqueous fuchsin. Magnification ×1000.

FIGS. 9 TO 12. Colonies on urea agar incubated for three days at 30°C. Magnification ×10.



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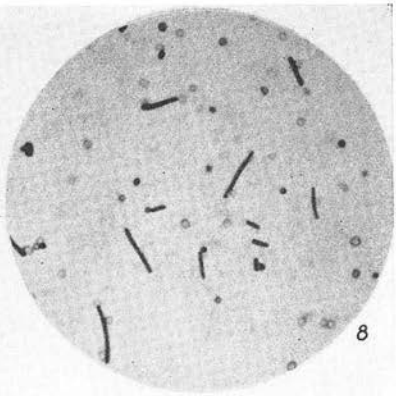

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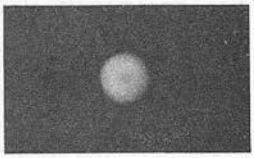

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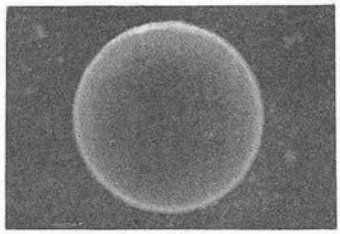

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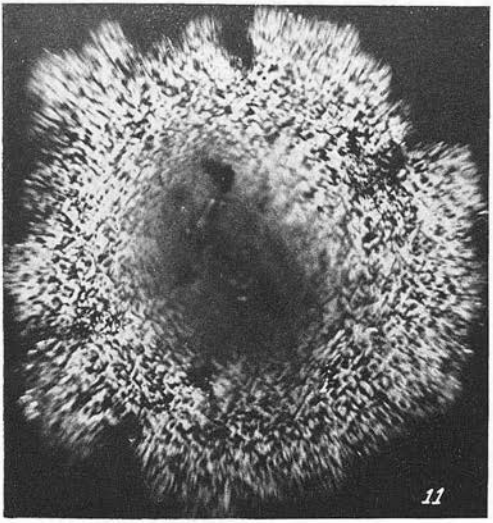

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AN INVESTIGATION OF THE BACILLUS PASTEURI GROUP

II. SPECIAL PHYSIOLOGY OF THE ORGANISMS

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In the first accounts of *Urobacillus Pasteurii* by Miquel (1889, 1898) the organism was described as having been cultivated in bouillon and peptone media containing various concentrations of urea. Beijerinck (1901) found that the bacillus would not grow on the ordinary laboratory media, but developed in bouillon containing 10 per cent urea, or an agar and gelatin to which 2 per cent urea and 0.3 per cent ammonium carbonate were added. He was unable to obtain growth of the organism in urea peptone solutions unless Chapoteau peptone, which was used by Miquel, was employed. Simple media containing, in addition to urea, asparagine and glucose, or ammonium salts of organic acids, did not support growth. Löhnis and Kuntze (1908) used bouillon, agar, and gelatin, each containing 2 per cent urea, for the cultivation of the organism, and confirmed Beijerinck's observations concerning the unsuitability of media which contained no urea or protein. It became generally accepted that the most important criterion for the recognition of *B. Pasteuri* was its inability to grow on ordinary media without added urea. Later work by Viehoever (1913), however, cast doubt on the necessity for urea, and this author observed growth of the organism in much simpler media than those used by Beijerinck and Löhnis and Kuntze. Viehoever described good growth in an ammonium carbonate mineral solution containing glucose and asparagine as the only organic compounds. In the same medium without glucose the organism was still capable of growing, but less vigorously. Viehoever added either urea, ammonium carbonate, or sodium carbonate to all the media he used.

There appears, therefore, to be considerable uncertainty concerning the environmental and nutritive requirements of *B. Pasteuri*, especially with regard to the necessity for urea. The function of urea in the metabolism of the organism has not been examined experimentally. It has, however, been established that the bacillus requires alkaline media, and a consideration of the literature suggests the possibility that urea merely serves to produce a strongly alkaline reaction through its breakdown to ammonium carbonate. This change would occur to some extent during the heat sterilisation of artificial media, and would later be completed by the urease activity of the cell.

THE EFFECTS OF UREA AND AMMONIA ON GROWTH

In this investigation it was observed that most of the strains usually failed to develop from light inoculations in media prepared from one per cent peptone and one per cent meat extract, and adjusted to pH 7.0 to 7.5. If the media were heavily inoculated, many strains initiated growth after incubation for several days, or several weeks, and having started, the organisms multiplied rapidly and the quantity of macroscopic growth frequently exceeded that formed on the same medium to which urea had been added. Evidently the heavy seeding enabled the cells to produce conditions suitable for their multiplication. When the organisms succeeded in growing in ordinary bouillon or agar, the cultures became distinctly alkaline, and the possibility that these substrates might be suitable for growth if made sufficiently alkaline was investigated. Tests were made with bouillon varying in alkalinity up to pH 8.5, but many strains failed to initiate growth at any reaction. Tests were then carried out with samples of bouillon of different reactions, to which 2 per cent urea was added. For this purpose, the urea in concentrated aqueous solution was sterilised by filtration and added aseptically to the tubes of bouillon, thus avoiding complications arising from the decomposition of urea by heat. Small and fairly uniform inoculations were made by thoroughly emulsifying growth from agar in M/15 phosphate buffer of pH 8, so that a definite but faint turbidity was produced, and transferring a loopful to each

tube of bouillon. The results were unexpected. Some strains failed to grow and others grew only after a long interval in the urea bouillon at pH 7.5 to 7.7. In the more alkaline bouillons growth occurred with less difficulty, but only after an appreciable lag, and certain strains failed to develop in any sample. In all cases, if growth started it progressed rapidly. Control inoculations of urea bouillon sterilised by intermittent steaming were made at the same time, and these invariably yielded prompt growth. It became evident that urea bouillon was converted into a satisfactory medium for *B. Pasteuri* only as a result of heating, and that the beneficial effect of steaming was only in part attributable to the alkaline reaction arising from a partial hydrolysis of urea. The use of different peptones (Bacto, Proteose and Witte), and of meat infusion instead of meat extract for preparing bouillon, did not influence the results.

The next experiments were planned to show whether the beneficial effects of heating urea bouillon arose from an alteration of the urea, the meat extract, or the peptone. Ordinary bouillon, consisting of 1 per cent peptone and 1 per cent meat extract, was prepared in the usual way, and the reaction was adjusted to pH 7.5 by the addition of NaOH. The bouillon was divided into three portions. The first was untreated, the second was adjusted to pH 8.8 by adding Na_2CO_3, and to the third 0.5 per cent commercial ammonium carbonate was added. The three portions were distributed in tubes and sterilised at 22 pounds. A 50 per cent aqueous solution of urea was also prepared. Of this, one portion was sterilised by filtration and a second was autoclaved at 22 pounds. Another urea solution of the same concentration was prepared by weighing the crystals aseptically, dissolving them in sterile water and placing the solution in flowing steam for fifteen minutes. The urea solutions were pipetted aseptically into tubes of the different bouillons in amounts which brought the concentration of urea to 2 per cent in each case. The reaction of all the media was then adjusted to pH 7.8 by adding sterile Na_2CO_3 or H_3PO_4. The different media were inoculated by transferring to each tube a loopful of a faintly turbid suspension of cells in phosphate buffer. Table 1

gives the essential features of one of the experiments in which an incubation temperature of 30°C. was employed, the intensity of growth at twenty-four hours being indicated by the number of + symbols. The table shows that heating the bouillon which had been made alkaline with Na_2CO_3 did not improve the medium. The beneficial effect obtained by the use of ammonium carbonate can therefore be attributed to the presence of ammonia in the medium and not to an alteration of the bouillon constituents produced by sterilisation at an alkaline reaction. The table also shows that heating the urea apart from the bouillon resulted in an improved medium, and that the most drastically heated urea gave the best result. This effect can be attributed to a partial conversion of the urea to ammonium carbonate at the high

TABLE 1

Growth in 24 hours at 30°C. in media containing 1 per cent peptone, 1 per cent meat extract and 2 per cent urea; pH 7.8

BOUILLON	UREA		
	Filtered	Steamed 15 minutes	Autoclaved
1. Ordinary; pH 7.5 before sterilisation...	−	−	+
2. Adjusted to pH 8.8 with Na_2CO_3 before sterilisation.....	−	−	+
3. Ammonium carbonate (0.5 per cent) added before sterilisation.....	+	++	+++

temperatures. The urea solutions were all tested for cyanates with $AgNO_3$ but the results were negative. These experiments were carried out with several strains, some of which grow more readily than others on media without urea. The results were approximately the same in the case of each strain except one. The latter, an organism which is exceptional in its ability to grow easily on ordinary media, was not influenced definitely by the presence of ammonia. The results of these experiments were confirmed by using agars instead of bouillons. Agar media produced clearer distinctions than bouillons for, in addition to showing the total amount of growth, they gave an indication of the relative number of cells in the inoculum which initiated growth on each medium.

It appeared to be certain that the ammonia requirement of the organisms is unusually high. Experiments were accordingly carried out to test the effect of adding ammonium salts to bouillon or agar. In the first tests the media, adjusted to pH 7.8 to 8.2, were made up to contain when completed 1 per cent peptone, 1 per cent meat extract, 2 per cent urea sterilised by filtration, and varying proportions of $(NH_4)_2SO_4$. The last two ingredients were added to the remainder after sterilisation. The tests again demonstrated that growth was considerably delayed in the absence of added ammonia, and they further showed that the addition of 0.2 per cent $(NH_4)_2SO_4$ to the media containing unheated urea permitted growth as readily as did urea bouillon or urea agar sterilised by steaming.

In the succeeding experiments, media containing different concentrations of $(NH_4)_2SO_4$ or NH_4Cl but no urea, were used. The ammonium salts in concentrated aqueous solution were sterilised separately and added to the alkaline media immediately before inoculation. Growth occurred most readily when about 1 per cent of either salt was added, and if the reaction was close to pH 8 the organisms developed almost as quickly as in the usual urea media. The optimum concentration of ammonia was not clearly defined: most of the strains examined appeared to grow with equal facility at any concentration between 0.5 and 2 per cent. The optimum for certain strains which decompose urea slowly appeared to be between 0.5 and 1 per cent of either the sulphate or the chloride. The maximum concentration of ammonia tolerated by the organisms is high, but in attempting to elucidate this factor the results were erratic and inconclusive. Wide differences were obtained with a single strain when different amounts of inoculum were used.

THE EFFECT OF REACTION

Having obtained a suitable medium which is not subject to violent changes in reaction as a result of growth, a study was made of the effects of hydrogen ion concentration. Bouillon with a high buffering capacity, containing 2 per cent peptone, 2 per cent meat extract and 1 per cent NH_4Cl, was used. In

order to obtain a medium which would remain clear when made alkaline, the bouillon was prepared by adjusting the reaction to approximately pH 9, autoclaving, filtering, and bringing the reaction back to neutrality, prior to adding the ammonium salt and sterilising. By adding sterile Na_2CO_3 or HCl aseptically to samples of the bouillon, several series of media varying in reaction were obtained. These were inoculated with dilute suspensions of the organisms, and observations were made for the appearance of growth during incubation.

For most strains the optimum reaction proved to be in the region of pH 9, but in the case of the least active urea-decomposing organisms it was nearer to pH 8. The active strains seldom grew at pH values below 7.5, while the inactive types usually produced visible growth at pH 7. In these experiments few strains developed on the acid side of pH 7; but on other occasions a number of strains were observed to grow in media as far from neutrality as pH 6.2 to 6.4, if heavy inoculations were made. The alkaline limit for growth appears to be inconstant even for individual strains: the vigour of growth, the composition of the medium, the temperature, and probably other factors seem to produce variations.

NUTRITIVE AND OTHER ASSOCIATED REQUIREMENTS

The growth of the organisms was tested in a considerable number of media. The most suitable substrates were prepared from bouillon containing comparatively high concentrations of peptone and meat extract. The use of at least 1 per cent peptone is especially important in the case of urea-containing media, in order to prevent cultures from becoming excessively alkaline. Optimal conditions for growth in bouillon were produced by adding 0.5 to 1 per cent NH_4Cl, and adjusting the reaction to between pH 8.5 and 9 after sterilisation. The organisms grew more profusely in this medium than in 2 per cent urea bouillon. They also developed earlier than in urea bouillon, especially if the latter had been allowed to stand for several weeks after preparation, so that its ammonia content was reduced. Alkaline ammonia-containing media cannot be sterilised

without deterioration, and urea media are therefore more suitable for general use. The comparative values of ammonia and urea suggest that the latter compound does not serve as an energy source in the metabolism of the organisms. It appears to be impossible, however, to investigate this question satisfactorily with the complex media which were found to be necessary in this work. Söhngen (1909) and Christensen (1910) stated that certain bacteria which decompose urea may utilise energy liberated by the reaction, but their findings were not supported by any decisive proof.

In nature, bacteria of this group seldom encounter media comparable in nutritive value to bouillon, and their ability to grow in poorer substrates was tested. In autoclaved soil, soil extract and dilute peptone solutions (0.1 to 0.01 per cent) the organisms were capable of growing, provided the reaction and the content of ammonia were suitable, but the amount of growth produced was meagre in comparison with that in bouillon cultures. A visible growth was never observed in synthetic solutions which contained glucose or salts of organic acids as carbon sources, and asparagine with the addition of either ammonia or urea as nitrogen sources. No further efforts were made to confirm the findings of Viehoever (1913) and Rubentschik (1925, 1926) that organisms of this group are capable of multiplication in synthetic media, for if bacteria are unable to produce a macroscopic growth in a transparent test solution, evidence of their proliferation obtained by counting methods is generally of uncertain value.

Observations which indicate how bacteria of the Pasteuri group may find in nature conditions suitable for growth were made from experiments in bacterial association. In protein-containing media to which neither ammonia nor urea were added, organisms of this group, while incapable of multiplying in pure culture, were able to proliferate actively in the presence of other bacteria which decompose the protein. Solutions of casein and gelatin (1 per cent) adjusted to pH 7.5 to 7.7 were inoculated with *B. mycoides* and with a small number of cells from a dilute emulsion of a Pasteuri agar culture. Several strains of the urea-decomposing organisms produced, after

incubation for some days, a densely turbid growth, distinguishable from the flocculent growth of *B. mycoides*, and by plating on urea agar, the number of viable cells of the former was found to be of the order of 10^{10} per ml. of culture. Growth of the urea decomposers took place more readily if they were inoculated into cultures of *B. mycoides* which had been incubated previously for seven to fourteen days so that the protein decomposition was already well advanced. In the tests with casein and gelatin the strains of *B. Pasteuri* which are especially dependent on an alkaline reaction and a high concentration of ammonia did not initiate growth easily, but these strains proved to be capable of developing on ordinary agar in association with other bacteria, such as organisms of the Coli group. When agar was used, the liquefied medium was inoculated with the urea decomposer, poured into a Petri dish, allowed to solidify, and then inoculated at one point with the second organism. After a period of incubation, the length of which depended on the strain used, colonies of the urea-decomposing bacillus appeared in the agar around and beneath the growth of the other organism, but not on the remainder of the plate. It seems probable that the ability of organisms of the Pasteuri group to grow in the presence of other bacteria depends on the production of ammonia by the latter.

The inability of *B. Pasteuri* to grow in the ordinary bacteriological media adjusted to neutrality has been considered an important diagnostic feature. The Pasteuri group does, however, comprise a series of types varying from the most active urea-decomposing strains which cannot be cultivated in ordinary neutral bouillon, to feebly ureaclastic organisms which grow readily from small inocula. A test with ordinary media does not therefore permit of a clear differentiation of the group, and it is further complicated by variations due to the amount of inoculum and, especially in the case of agar, by the evolution of ammonia from other cultures in an incubator. Neutral one per cent peptone is of greater differential value than bouillon. Of the strains examined in this work only three develop easily in a peptone solution if the medium is lightly inoculated. Milk and potato, probably on account of their reaction, do not support the growth of any strain unless they are inoculated fairly heavily.

THE FORMATION OF NITRITES

In seeking an explanation of the unusually high ammonia requirement, the possibility that the organisms utilise ammonia as a source of energy was considered. Viehoever (1913) has claimed that bacilli of this group are capable of oxidising ammonia to nitrous acid, and that they may multiply to some extent in a solution in which ammonium carbonate constitutes the only source of carbon and nitrogen. Should this claim be substantiated, it would be of more than theoretical interest, for the invariable occurrence of these organisms in farm manure would account for a loss of nitrogen through nitrite formation and subsequent denitrification. Several of the strains used in this work, when cultivated in peptone water containing 0.5 per cent ammonium sulphate, produced appreciable quantities of nitrites as shown by using the Griess-Ilosvay reagents, while other strains did not. The strains which formed nitrites are all nitrate reducers and those which gave negative or doubtful tests do not reduce nitrates. It seemed probable, therefore, that the nitrites were formed by the reduction of traces of nitrates in the medium. There are numerous records of the formation of small amounts of nitrous acid from ammonia or from organic matter by heterotrophic microörganisms, but it should be pointed out that experimental work on this problem is particularly subject to error. The chief difficulties arise from the use of highly sensitive reagents for nitrites, the lack of comparably sensitive nitrate reagents, the frequent occurrence of nitrates as impurities in the ingredients of culture media, and the presence of oxides of nitrogen in the atmosphere, especially in the proximity of Bunsen burners. Without describing the exploratory work, it may be stated that after trying a considerable number of media and after preparing the purest ammonium salts, it was found that strains of *B. Pasteuri*, which readily formed nitrites when tested in the ordinary way in ammonia peptone solutions, could be cultivated at 30°C. for periods up to seven days under conditions permitting of excellent growth, without a trace of nitrous acid being formed. In the final experiments media were used immediately after preparation, the usual flaming of culture con-

tainers was omitted, and the cultures were incubated in a stream of purified air. It was concluded that *B. Pasteuri* is incapable of oxidising ammonia. The experiments also suggest that similar work with other heterotrophic bacteria requires the most rigid control.

SUMMARY AND CONCLUSIONS

Most of the organisms which may be placed in the *Bacillus Pasteuri* group require alkaline media containing ammonia. In the case of strains which bring about the most rapid decomposition of urea and are incapable of growing in ordinary neutral media, optimal conditions for growth in bouillon are produced by adding about 1 per cent NH_4Cl and adjusting the reaction to about pH 9. Strains which produce a weaker action on urea are less dependent on ammonia and an alkaline reaction, but few of them grow readily from small inocula on the ordinary bacteriological media. The most suitable substrates for cultivating the organisms contain urea from which the necessary ammonia is formed during sterilization and later by the urease activity of the bacteria. The organisms grow most readily in media containing comparatively high concentrations of peptone and meat extract. A visible growth has not been observed in synthetic solutions. In media containing ordinary bouillon or a protein, the organisms, while incapable of growth in pure culture, may proliferate actively in the presence of other bacteria. The appearance of nitrites in ammonia-containing cultures may be attributed to one or more of several circumstances, but not to an oxidation of ammonia by the organisms.

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AN INVESTIGATION OF THE BACILLUS PASTEURI GROUP

III. SYSTEMATIC RELATIONSHIPS OF THE GROUP

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For many years the literature dealing with the active urea-decomposing bacteria of soils and manures (reviewed by Löhnis, 1910, and Waksman, 1931) has been overburdened with the names of imperfectly defined species. A considerable proportion of the organisms appear to belong to the *Bacillus Pasteuri* group, which has been described (Gibson, 1934) as a series of ureaclastic spore-forming bacilli which either require or exhibit a preference for alkaline media containing ammonia. An attempt has been made to collect the existing type cultures of organisms which, according to their original descriptions, might be expected to be related to *B. Pasteuri*, and several strains were secured. This paper contains a brief description of the type cultures, proposals concerning the classification of the Pasteuri group, and an account of work pertaining to its differentiation from other bacteria.

INVESTIGATIONS OF TYPE CULTURES

The results of the study of type cultures and of their comparison with recently isolated strains are given in this section. In addition, a short historical account of each type is presented, in order to evaluate its claim to recognition as a species and to clarify the confused nomenclature of the organisms.

Bacillus Pasteuri (Miquel) Migula

This organism, originally described as capable of decomposing 3 grams of urea in a liter of culture per hour, has been regarded

generally as the most distinctive species of the group. The description of *Urobacillus Pasteurii* given by Miquel (1888–89; 1889–90, p. 13) is unsatisfactory according to modern standards, but the type of organism with which he dealt may be identified with reasonable confidence by its exceptional power of decomposing urea. Migula (1900) changed the name of the organism to *Bacillus Pasteuri* without amplifying its characterization. Miquel's strains appear to have been lost, but Beijerinck (1901; 1902) described an organism, *Urobacillus Pasteurii*, which he considered identical with Miquel's type. Löhnis and Kuntze (1908) provided a still more complete description, under the specific name of Migula, based on one of Beijerinck's cultures and twelve strains of their own isolation.

Three type cultures of *B. Pasteuri* were examined in this work. A strain of Beijerinck's was received from Prof. A. J. Kluyver. It is not certain that this is the strain described in Beijerinck's classical paper, but it conforms in every way to his description except in a decreased ability to form spores. Strain A1 and strain A4a, described by Löhnis and Kuntze, were received from Prof. F. Löhnis. The latter strains now appear to be incapable of forming spores, and they produce the "rougher" types of colony (Gibson, 1934, pp. 302, 306) in which the organisms frequently grow as filaments. All three strains liquefy gelatin comparatively rapidly and reduce nitrates. Beijerinck's strain is identical with a number of the most active urea-decomposing types among the recently-isolated organisms; and Löhnis and Kuntze's strains are identical with some of the variants which were produced artificially in this work. The strains received from Löhnis both require about five days at 30°C. for complete decomposition of 2 per cent urea in bouillon, indicating a considerable reduction in urease activity in the case of strain A4a since 1908.

Urobacillus Leubei Beijerinck

Beijerinck's (1901) culture, purchased from the Kral museum, proved to be a short, stout rod which forms ovoid to elliptical spores in fusiform sporangia. Its action on urea is comparatively

feeble, and it can be induced to grow on ordinary agar. The organism reduces nitrates but does not liquefy gelatin within five months. It is identical in every respect with 8 of the recently isolated strains, and it can be distinguished from *B. Freudenreichii*, as described by Löhnis (1905), only by its inability to liquefy gelatin.

Miquel (1904) used the name *Urobac. Leubei* for Leube's (1885) *Bacterium ureae*. A culture, bearing the designation *B. ureae* Leube No. 1654, was obtained from the Lister Institute. In a private communication Dr. St. John-Brooks stated that this culture was derived from a transfer of Beijerinck's *Urobac. Leubei*. It proved to be a spore former which differs in many respects from organisms of the Pasteuri group, including an inability to decompose urea. As this bacillus has been used for biochemical investigations by other workers (Birkinshaw et al., 1931) under the name *B. ureae*, it should be put on record that it shows no resemblance to its parent strain in Vienna or to Leube's (1885) *Bacterium ureae* or to Günther's (1906) *Bacillus ureae*.

Bacillus Freudenreichii (Miquel) Migula

Incomplete descriptions of *Urobacillus Freudenreichii* were made by Miquel (1889–90, pp. 367, 488) and later by Miquel and Cambier (1902). The organism was renamed *Bacillus Freudenreichii* by Migula (1900), but was properly described for the first time by Löhnis (1905) who acted on the assumption that cultures of his isolation were probably identical with Miquel's bacillus. Bierema (1909) later isolated strains of *Bacillus pumilus* and organisms intermediate between *B. pumilus* and *B. Freudenreichii*, all of which decomposed urea. This work led Löhnis (1909) to regard *B. Freudenreichii* as a variety of *B. pumilus*. It should be pointed out, however, that the bacilli which Löhnis and Bierema recognized as *B. pumilus* were probably of a type different from the Kral strain which Lawrence and Ford (1916) examined.

A culture which is reputed to be descended from one of the original strains of *B. Freudenreichii* was received from Prof. F. Löhnis, and two cultures derived from Bierema's *B. pumilus* strains from Dr. H. Glathe. All proved to be of the Mycobacte-

rium type and differed markedly from the original sporing bacilli. Similar observations on these cultures were made by Löhnis and Smith (1923). The strains designated "*B. pumilus*" differed from the "Freudenreichii" culture in several respects, including a feeble urease activity. It has been impossible to secure an authentic culture of *B. Freudenreichii* for comparative study, but it is certain that the strains described by Löhnis belong to the Pasteuri group.

Bacillus probatus Meyer et Viehoever

Viehoever (1913) gave a lengthy account of this organism based on a single culture. He also examined stock strains from other laboratories and concluded that Beijerinck's original cultures of *Urobac. Pasteurii* and *Urobac Leubei,* and one of the cultures of *B. Pasteuri* described by Löhnis and Kuntze, were identical with his organism. Having reached this conclusion, Viehoever had no justification for introducing the new name *B. probatus,* especially as he himself pointed out (p. 213) that Löhnis adequately described the urea decomposers with which he dealt. Few would consider the characterization of *B. probatus* superior or even equal to that given by Löhnis and Kuntze for *B. Pasteuri.* The name proposed by Viehoever is therefore a homonym which must be rejected. A culture of *B. probatus* was obtained from the Kral collection. The organism is a small rod which forms slightly ovoid terminal spores. It produces a comparatively slow decomposition of urea and adapts itself to ordinary media more easily than the majority of organisms in the group. Gelatin is liquefied comparatively rapidly and nitrates are reduced.

CLASSIFICATION OF THE GROUP

In the first paper of this series (Gibson, 1934) the conclusion was reached that a natural subdivision of the Pasteuri group, on the basis of the commonly used diagnostic tests, is not possible. All distinctive strains are connected by numerous transitional forms and a precise definition of species is out of the question. It was indicated, however, that the organisms may be divided roughly into three types according to the shape of spore and

urease activity, the latter being associated with conditions of growth. The three types established on these criteria each contain an extreme form; and since the organisms representing the extremes are unmistakably different, it appears to be expedient to divide the group into three species. *B. Pasteuri* and *B. Freudenreichii* fit into this classification, but a new name is required for the third species which is the most prevalent type of the group in soils. The use of urease activity as a differential criterion necessitates the selection of some arbitrary standard, and it is suggested that the complete decomposition of the urea in bouillon containing 2 per cent of this compound within forty-eight hours be taken as the characteristic of the most active organisms. The medium (Gibson, 1934) should be used soon after preparation and should be inoculated with a loopful of liquid culture, but great refinement of the test is impossible and is scarcely warranted by the behavior of the organisms.

The three species into which the Pasteuri group may be divided are differentiated as follows:

1. *Bacillus Pasteuri* (Miquel) Migula. Spores spherical or slightly ovoid and terminal or sub-terminal. All the urea in 2 per cent urea bouillon decomposed within forty-eight hours at the optimum temperature. No growth in ordinary neutral media. Best growth in alkaline (about pH 9) media containing comparatively large quantities of ammonia (about 1 per cent NH_4Cl).

2. *Bacillus Loehnisii* n. sp. Spores spherical or slightly ovoid and terminal or sub-terminal. Decomposition of 2 per cent urea in bouillon does not reach completion within forty-eight hours at optimum temperature. Generally capable of growing in ordinary neutral media. Best growth in moderately alkaline (pH 8 to 8.5) media containing ammonia (about 0.5 per cent NH_4Cl).

3. *Bacillus Freudenreichii* (Miquel) Migula. Spores ovoid to elliptical, formed in a central or excentric position. Indistinguishable from 2 in urease activity or conditions of growth.

Each of the three species contains strains which exhibit any combination of characters, other than those used in differentiating the species, within the limits of the descriptions given in the first paper of this series. There is perhaps one exception to this state-

ment: among the strains examined, those with the lowest temperature maxima (under 30°C.) all belong to *B. Loehnisii*. In the case of *B. Pasteuri* and *B. Freudenreichii*, the characterizations proposed here involve a slight modification and extension of the original descriptions and of the emended descriptions of these species given by Löhnis. Beijerinck's (1901) *Urobac. Leubei* is regarded as a synonym of *B. Freudenreichii*. The latter name is preferred, partly because it was used earlier (1889–90) by Miquel and partly because it was employed by Löhnis (1905) in the first adequate description of an organism of this type. Löhnis and Kuntze (1908) included in the species *B. Pasteuri* strains which acted slowly upon urea. There is ample justification for this attitude, but in any subdivision of the group the highly specialized organisms which correspond to the *Urobac. Pasteurii* of Miquel and Beijerinck should be differentiated from the others. *Urobac. psychrocartericus* and *Urobac. hesmogenes*, described by Rubentschik (1926), are undoubtedly strains of this group since the descriptions of these organisms contain nothing which distinguishes them from *B. Pasteuri* as defined here. The possibility of using *Urobac. psychrocartericus* to comprise strains with low temperature requirements has been considered but appears to be impracticable. Of the strains examined, those exhibiting a maximum temperature below that of Rubentschik's organism belong to the *B. Loehnisii* type and they differ among themselves in their action on gelatin and nitrates and in other features.

The classification of the Pasteuri group proposed here is recognized as distinctly artificial. It does not even possess the merit of exactness, and consequently the diagnosis of many strains will be doubtful. Other defects are the use of such criteria as urease activity, which may become reduced in pure cultures, and of spore formation, which may disappear entirely in laboratory cultures and their variants. However, the classification may be of some value from the practical standpoint and it appears to be the best that can be devised at the present time.

A discussion of the Pasteuri group would be incomplete without a reference to a number of previously described organisms, cultures of which appear to be unobtainable. *Bacillus ureae* II and III of Burri et al. (1894), *Urobacillus Duclauxii* Miquel (1889–90,

pp. 53, 122, 145) and *Urobacillus Maddoxii* Miquel (1890–91, pp. 275, 305) were imperfectly described. Although subsequent writers (Söhngen, 1909; Düggeli, 1915; Kusnetzow, 1930) have undertaken to diagnose strains of their own isolation by means of Miquel's descriptions, this could not be done with certainty, and the names of these organisms should not be used in future. It may be indicated here that the descriptions of Burri and Miquel contain nothing which is incompatible with the organisms belonging to the Pasteuri group.

Considering the tendency of many strains in this group to conceal or to lose their ability to form spores, it is not unlikely that some of the urea decomposers which have been described as non-sporers also belong to the group. This statement may apply to *Bacillus ureae* I Burri et al. (1894), *Urobacillus Jakschii* Söhngen (1909), and to *Bacterium ureae* Leube (1885), an organism which was renamed *Urobacillus Leubei* Miquel (1904) and later *Bacillus ureae* Günther (1906). All these specific names, and also others due to Miquel and Cambier, should however be discarded for the descriptions are insufficient and cultures do not appear to be available. The detailed descriptions given by Rubentschik (1926) of *Urobacterium amylovorum, Urobact. citrophilum,* and *Urobact. aerophilum* show that these organisms cannot be distinguished from the Pasteuri group except by inability to form spores.

The organisms carefully described by Geilinger (1917) undoubtedly belong, with the possible exception of the strain "Erde b," to the Pasteuri group. Geilinger's paper is particularly valuable for it provides a detailed account of the variations in cultural characteristics of different strains, and shows how individual organisms may embody the characters of two or more previously described species. A few of Geilinger's organisms proved to be facultative anaerobes, growing actively and decomposing urea in the complete absence of oxygen.

DEMARCATION OF THE GROUP

The descriptions given in the first paper of this series appear to differentiate the Pasteuri group from all other species of ureaclastic bacteria which have received an adequate characterization

in the past, and also from several undescribed organisms which were isolated in this work. From bacilli which do not decompose urea, the group is distinguished principally by the urease activity of its members and by the beneficial effects of alkalinity and ammonia on their growth. There are several references in the literature (especially Löhnis, 1910, and Lehmann and Neumann, 1927) which suggest that in certain bacteria the ability to bring about an active decomposition of urea may be found in some strains but not in others. The possibility should therefore be considered that organisms which have been included in the Pasteuri group in this series of papers are merely varieties of bacteria which do not hydrolyse urea. This possibility appears to be remote except in the case of those members of the group which possess feeble ureaclastic powers and the ability to grow on ordinary media. Certain strains of this type resemble *B. sphaericus* Meyer et Neide [*B. pseudotetanicus* (Kruse) Migula] as described by Neide (1904), Ford (1916) and Cunningham (1931), and other strains approach in their characters *B. tritus* Batchelor (1919). Cultures of *B. sphaericus* were obtained from Kral (1 strain), from Ford (1 strain), and from Cunningham (9 strains), and were tested in urea bouillon. Ten strains produced no action on urea, but one of Cunningham's cultures brought about a feeble decomposition. This latter organism can be distinguished from other strains of *B. sphaericus* only by its urease activity, and it is identical with two strains isolated in this work, which, on account of their slow action on urea and their ready growth on ordinary media, had been regarded as border-line types of the Pasteuri group. The existence of such organisms indicates the absence of a natural boundary, but it seems desirable for practical reasons that they should be excluded from the Pasteuri group and regarded as ureaclastic strains of *B. sphaericus*. Their growth on artificial media is not promoted by alkalinity and the presence of ammonia, and their exclusion appears to leave intact this important criterion of the Pasteuri group.

To discover possible relationships with non-ureaclastic bacteria, a considerable number of cultures isolated in this work, but found to be incapable of decomposing urea, were investigated further.

None of them is of the *B. sphaericus* type, and the great majority are differentiated from the Pasteuri group by at least one feature other than their inability to hydrolyse urea. A few of the organisms, apparently belonging to undescribed species, require alkaline media containing ammonia, and they cannot be distinguished with certainty from strains of the Pasteuri group except by their inactivity towards urea. It therefore appears necessary to fix arbitrarily the limits of the Pasteuri group so as to exclude bacilli which fail to decompose urea, and also those which do not exhibit a preference for special media, although they possess no other differential feature.

It may be questioned whether the ability to bring about an active ammonification in urea bouillon is a feature of importance from the systematic standpoint, for every grade of urease activity is found among microörganisms. The titration of urea-containing cultures of many bacteria and fungi may yield inconclusive or negative results, whereas the organisms may be shown to contain urease by the more delicate test of adding a neutral urea solution containing phenol red to a neutral emulsion of cells and incubating the mixture, along with the necessary controls, at 45°C. The active decomposition of urea is therefore an arbitrary character, but probably in no greater degree than many other criteria used in classification. For the Pasteuri group it serves at least to bring together a large assemblage of strains among which there appear to be no natural divisions. In the Pasteuri group the necessity for ammonium carbonate in the medium and resistance to high concentrations of this compound vary roughly with the urease activity of the organisms, but the latter property does not appear to be a consequence of a special tolerance of ammonia. In this work, a considerable number of bacteria, which do not produce a titratable alkalinity in urea bouillon, have been found to tolerate higher concentrations of ammonium carbonate than the weaker ureaclastic strains of the Pasteuri group.

THE GENERIC TERM UROBACILLUS

Urobacillus, introduced by Miquel (1888–89), has been used in a generic sense by Krogius (1890), Beijerinck (1901), Liebert (1909),

Orla-Jensen (1909), Söhngen (1909), Christensen (1910), Düggeli (1915), and Rubentschik (1926). In most cases the term was applied to any rod-shaped organism which decomposed urea actively, but Orla-Jensen proposed that the name be reserved for the spore-forming types. Rubentschik used the term *Urobacterium* for his non-sporing ureaclastic organisms.

If the prevailing tendency to multiply bacterial genera should persist, it is possible that the question of the recognition of a genus *Urobacillus* may be raised. It would be definitely unsatisfactory to include non-sporing organisms under this designation, for strong urease activity has been observed in various members of the Proteus, Coli-Aerogenes and Fluorescens groups, and in several chromogenic and other non-sporing bacteria. Among spore-forming bacilli the situation is almost as complicated, for the ability to decompose urea has been found in certain anaerobes (Tissier and Martelly, 1902), in occasional strains of *B. mycoides* and *B. megatherium* (Löhnis, 1910; Düggeli, 1915; Goeters, 1927), and in other organisms which do not require special media (Rochaix and Dufourt, 1910; Gibson, unpublished results). The genus *Urobacillus* might be restricted to include only the Pasteuri group and other ureaclastic aerobic spore formers which require or prefer alkaline, ammonia-containing media, but it is doubtful if these organisms constitute a series sufficiently well defined to merit generic status.

SUMMARY

Type cultures of organisms of the *B. Pasteuri* group were examined and compared with recently-isolated strains. The group may be divided into three moderately distinctive species: (1) *B. Pasteuri*, (2) *B. Loehnisii* n. sp. and (3) *B. Freudenreichii*. Spores are spherical or slightly ovoid in (1) and (2) and ovoid to elliptical in (3). Urea is decomposed rapidly by (1) and slowly by (2) and (3). The ability to decompose completely the urea in 2 per cent urea bouillon within forty-eight hours is suggested as the standard of urease activity distinguishing (1) from (2) and (3). Growth in ordinary neutral media is usually possible only for (2) and (3). Each species contains strains dissimilar in several

features and each is connected to the others by transitional forms. *Urobacillus Leubei* is regarded as a synonym of *B. Freudenreichii*. *Urobac. psychrocartericus* and *Urobac. hesmogenes* are probably synonyms of *B. Pasteuri*; and several other previously named bacteria appear to belong to this group. *B. sphaericus* and other organisms closely related to the Pasteuri group cannot be differentiated from the latter except by their inability to decompose urea or by their failure to show a preference for alkaline media containing ammonia. The suggestion is put forward that there is no group of urea-decomposing bacteria sufficiently well defined to deserve the generic term Urobacillus.

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T. Gibson:

An investigation of Sarcina ureae, a spore-forming motile coccus

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(From the Department of Bacteriology, College of Agriculture, Edinburgh.)

An investigation of Sarcina ureae, a spore-forming, motile coccus.

By

T. Gibson.

(Eingegangen am 9. Dezember 1934.)

The existence of *Cocci* which are furnished with flagella and are capable of active motility is widely recognised. *Migula* (1900), *Zettnow* (1918), *Lehmann* and *Neumann* (1927), and *Hucker* and *Thatcher* (1929) describe a number of examples. Records of spore formation among *Cocci* are on the other hand rare, the most notable being those by *Hauser* (1887) and *Lehmann* and *Neumann* (1927) for *Sarcina pulmonum Virchow*, and those by *Beijerinck* (1901) and *Ellis* (1902) for *Planosarcina ureae Beijk.* At the present time there appears to be a widespread belief that the early observations on these *Sarcinae* are not trustworthy. For example, *Bergey* (1934, p. 29) states that in the *Coccaceae* "endospores are not formed", and in the description (p. 105) of *Sarcina ureae*[1] the following statement appears: "Typical endospores absent, though heat resistant spore-like bodies have been described". This standpoint may be attributable to the fact that cultures of *Beijerinck*'s *Sarcina* which have been preserved do not readily form spores unless suitable conditions are provided, while it is not clear that the isolation of the organism has been repeated.

A simple and effective method of isolating *Sarcina ureae* from soils was devised recently, and a description of the procedure is given in this paper. The available information concerning this interesting organism is somewhat fragmentary, and it also appears to be desirable to give an account of its characters which is based on an investigation of a number of strains obtained from different sources.

Isolation of pure cultures.

Flasks containing 100 ccm agar (2 % agar, 1 % peptone, 1 % meat extract, p_H 7—7,5) are prepared, and immediately before use the medium is melted, 10 g crystalline urea are added, and after steaming for 10 minutes the agar is cooled and used for plating. Plates may be prepared from quantitative

[1] *Löhnis* (1911, p. 138) proposed that *Beijerinck*'s *Planosarcina* should be referred to as *Sarcina ureae*. Accordingly the emended name should be attributed to *Löhnis* and not to *Bergey* et *al.*

dilutions in the usual way, but recognition of the colonies of the organism is facilitated, if 0,1 ccm quantities of the soil dilutions are spread on the surface of previously-hardened and dried agar. Heating of the soil dilutions at 80° C may be adopted, but this procedure has little or no advantage as the majority of organisms which develop on the medium are spore-forming bacteria. In common with other urea-decomposing bacteria, the *Sarcina* is capable of growing in the high concentrations of urea and of ammonium carbonate which is formed from the latter, while bacteria which do not hydrolyse urea are inhibited. If the plates are incubated at 30° C, or preferably at 22° C, the colonies of *Sarcinae* which develop may be recognised by their slightly yellowish colour and by their coarsely granular structure when examined by low power and transmitted light.

It is probable that the difficulty which has been encountered hitherto in repeating *Beijerinck*'s isolation of the organism is due to the unsuitability of urea-containing solutions for selective cultures. A soil which contains this organism in numbers of the order of 10000 per g as shown by plating, was examined frequently by inoculating several liquid media with varying amounts of soil, including high dilutions, and incubating at various temperatures, but the *Sarcina* was never detected when the cultures were plated.

Fifteen strains of the organism were isolated by replating colonies which developed on plates inoculated with soil. The pure cultures, upon which the following description is based, exhibited a marked uniformity in their characters.

Occurrence in soils.

Sarcina ureae has been found to occur in considerable numbers in a rich garden soil (p_H 7.3, loss on ignition 15.3 %) and in four cultivated field soils varying in reaction between p_H 5.9 and 7.3. The results of plating measured dilutions indicated that these soils contained between 10000 and 20000 *Sarcinae* per g. On plates prepared from the garden soil colonies of the *Sarcina* were much less numerous than those of the *Pasteuri group* and other urea-decomposing bacteria, but in the field soils *S. ureae* produced about fifty per cent of the colonies which developed readily upon the agar. A series of strongly acid (p_H 4 to 5.5) hill soils which are rich in organic matter were also examined by direct plating, but the *Sarcina* was not detected in any of the samples. The prevalence of the organism in fertile soils suggests that it plays an active part in the decomposition of urea. It is likely, however, that its importance in this connection is less than that of the *Pasteuri group*. The urease activity of the organism is comparatively feeble, and stained films (*Cholodny* method) indicate that *Bacilli* multiply more rapidly than *Sarcinae* when soils are treated with comparatively large amounts of urea.

Morphology and heat resistance.

An extensive account of the morphology of *Sarcina ureae* was given by *Ellis* (1902), but it appears to be desirable to provide further information, particularly with regard to the formation of spores.

The organism is a definite *Coccus*. Occasional ovoid cells may be found in old cultures, but to no greater an extent than in cultures of other *Sarcinae* or of *Staphylococci*. Stained cells usually measure about 1.3 μ in diameter, but may vary from 1 to 2.5 μ. Tetrads are regularly produced, but larger packets are formed more rarely. The vegetative cells stain by *Gram*'s method but they are easily decolorised. Individual cells and packets are actively motile, the flagella being peritrichous. Illustrations of the flagella have been published by *Beijerinck* (1901), *Ellis* (1902), *van Niel* (1923), and by *Hucker* and *Thatcher* (1929). Cultures on agar are more vigorously motile than those in liquid media.

Spherical spores 0.8 to 1 μ in diameter are formed in a central position and occupy most of the cell interior. They tend to remain covered by the membrane of the sporangium for a considerable time, and consequently they are frequently observed in groups of four. These bodies cannot be regarded as anything other than true endospores. In the living condition they are more refractile than the vegetative cells, and in smears stained with an aqueous dye they exhibit a clearly-defined margin and an unstained interior, an appearance which may be differentiated readily from that of the faintly-staining cells found in old cultures of any *Coccus*. Further, the spores may be differentially stained by spore-staining methods, and their heat resistance is much greater than that of the vegetative organisms. The spores of all the recently-isolated strains survived exposure at 100° C and six of the strains tolerated this temperature for 5 minutes. Vegetative cells free from spores may be obtained by cultivating the organisms in liquid media or by making a series of daily transfers on an agar which induces an abundant growth, an incubation temperature of 30° C being used. The majority of the organisms in the resulting cultures are destroyed by heating at 65° C for 15 minutes, although when dense suspensions are used a few cells may survive exposure at 70° C for 15 minutes. In the tests from which these conclusions are drawn the organisms were heated in neutral bouillon containing 0.25 % NH_4Cl. Sufficient Na_2CO_3 to produce a reaction of p_H 8 to 8.5 was added to the tubes after cooling.

Factors which influence spore formation were investigated. The experiments indicated that spores are formed most abundantly on solid media which contain ammonium salts and sub-optimal concentrations of other nutrients; the reaction of the media should approach the limiting acidity for growth, and the incubation temperature should

not exceed 22° C. Rapid and extensive spore formation was observed on an agar, with a p_H value of 6.8 to 7, which contained 0,5 % peptone, 0,5 % meat extract and 0,5 % NH_4Cl. Some strains did not produce spores in large numbers, and there appeared to be a tendency for the spore-forming capacity to become reduced in artificial cultures.

Conditions of growth.

The organism is an aerobe, the colonies in agar shake cultures being restricted to the uppermost layer. At 30° C growth and urea decomposition occur slightly earlier than at 22° C, but the growth is ultimately more profuse at the lower temperature. The maximum temperature is under 37° C. Ordinary neutral media support growth, but alkaline media containing ammonia and comparatively high concentrations of peptone and meat extract permit of earlier and more abundant growth. The optimum reaction, as judged by the first appearance of turbidity in peptone bouillon containing 0.5 % NH_4Cl, is about p_H 8.8. In the same medium the organisms failed to proliferate at p_H values above 9.4 or below 6.4. The organisms were observed to grow on an agar containing glucose, asparagine, NH_4Cl and mineral salts, with a p_H value of approximately 8.5, but they appeared to be incapable of developing from small inocula in a solution of the same composition containing no agar. Growth did not occur on the glucose-asparagine-agar if the ammonium salt was omitted.

Cultural and biochemical characters.

The strains investigated appeared to be completely uniform in their cultural and biochemical characters and no exceptions to the following description were observed.

Colonies on agar and gelatin. Grey, round, becoming opaque; microscopically a uniform coarse granulation. Old colonies generally become slightly yellowish or brownish, and they become slightly irregular when sparsely distributed.

Agar slope. Grey, opaque, glistening growth confined to the inoculated area; generally becoming slightly yellowish in old cultures. — *Glucose agar stab*. Grey, glistening surface colony, becoming whitish or yellowish in the centre and slightly irregular in outline. Stab growth restricted to upper layers.

Gelatin stab. Grey or yellowish-grey, slightly irregular colony with glistening or dull surface. Stab growth thread-like or beaded. Softening or liquefaction of the medium may occur after several weeks or several months, but other samples of gelatin remain solid for over 12 months.

Bouillon. Turbid and, later, an easily dispersed sediment. A granular precipitate may be formed on the walls of the tube. — *Potato*. No growth on acid potato. A brownish growth develops on potato made alkaline with Na_2CO_3. — *Milk*. No change or (only if heavily inoculated) alkalinity after several weeks.

Formation of acid from glucose, diastatic action and indole- and H_2S-production are all negative. Nitrates are reduced to nitrites and urea is slowly decomposed.

Classification.

Transfers of *Beijerinck*'s original strain of *Planosarcina ureae* were received from Professors *F. Löhnis* and *A. J. Kluyver*. They were found to correspond in every way with the description given above. At first it appeared that the ability to form spores had been lost during the long period in which these strains have been maintained in artificial culture. Later, when the optimal conditions for spore formation had been worked out, both strains readily produced heat-resistant spores, although not in the profusion characteristic of some of the freshly-isolated strains.

Several descriptions of sarcinae are to be found in the literature which indicate that the organisms are very similar to, if not identical with, *Sarcina ureae*. The descriptions of *Sarcina pulmonum* given by *Migula* (1900) and by *Lehmann* and *Neumann* (1927) do not contain any definite differentiation of the two types except that the former author mentions neither spore formation nor motility. According to *Migula S. pulmonum* decomposes urea. The organism appears to have been isolated by several workers from the respiratory tract, chiefly in cases of phthisis, and this circumstance would indicate that it possesses a higher optimum temperature than *S. ureae*. A culture labelled ‚*Sarcina pulmonum Hauser*' was obtained from the *Kral* collection, but it proved to be a pleomorphic and chromogenic *Bacillus* which showed no resemblance to *Hauser*'s (1887) *Sarcina*.

Sames (1898) published a good description of a motile, urea-decomposing *Sarcina* which he isolated from liquid manure. It grew much better on alkaline media than on the usual substrates, and it appears to correspond in every way to *S. ureae* with the exception that spore formation was not observed. It is not unlikely that the production of spores by a true *Coccus* might pass unnoticed, especially if sporulation was suppressed by cultivation on alkaline media, and it may be concluded that this organism, which was later named *Sarcina fimentaria Lehmann* and *Neumann* and *Planosarcina Samesii Migula*, is probably identical with *S. ureae*.

Rubentschik's (1926) description of *Urosarcina psychrocarterica* shows that this organism closely resembles *S. ureae*. It was not observed to form spores, but, in other respects, the only recorded feature which might differentiate it from *S. ureae* is the production of an orange-yellow growth on the surface of stab cultures. This description of colour may be questioned in view of the fact that the growth on potato was described as dark yellow. In the absence of further evidence, particularly with regard to the organism's inability to form spores, it is doubtful if *Rubentschick*'s *Sarcina* should be accepted as a valid species[1].

Sarcina ureae is of considerable interest in systematic bacteriology. It is evidently another example of those connecting links which are so

[1] Unfortunately we have been unable to obtain cultures of any of *Rubentschik*'s new species of urea-decomposing bacteria.

frequently found to invalidate exact definitions in this subject. The general characters of the organism are those of a *Sarcina* rather than those of a sporing *Bacillus*, and there appears to be no reason for its wide separation from the non-sporing *Sarcinae*. If this conclusion be accepted it necessitates the recognition of spore-forming organisms among the *Coccaceae*. The possibility of creating a new genus to comprise the spore-forming *Sarcinae* may be suggested. There is probably as much justification for this suggestion as there exists for the recognition of several generic names now being used for different types of *Cocci*. *Beijerinck*'s (1901) paper indicates the occurrence in soils of organisms which may be still more difficult to place in a classification of the bacteria. The organisms referred to were described as being morphologically intermediate between *Sarcinae* and *Bacillus megaterium*. These observations do not appear to have received the attention which they deserve, for a study of the organisms might be expected to yield important results bearing on bacterial classification.

Summary.

A simple and effective method of isolating *Sarcina ureae* (*Beijerinck*) *Löhnis* from soils consists in direct plating on an agar containing 10 % urea. The organism was found to occur in numbers between 10000 and 20000 per g in fertile soils. Fifteen recently-isolated strains were found to be identical with a strain preserved by *Beijerinck*, and a description is given of their morphological, physiological and cultural characters. The organism is a definite *Coccus*. Spores, which tolerate exposure at 100^0 C for periods up to 5 minutes, are formed readily on an agar with a reaction of p_H 6.8—7, containing ammonium salts and sub-optimal concentrations of other nutrients. *Sarcina pulmonum Virchow*, *Sarcina fimentaria L.* et *N.* (*Planosarcina Samesii Migula*) and *Urosarcina psychrocarterica Rubentschik* are similar and possibly identical types.

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The Urea-decomposing Microflora of Soils.

I. Description and Classification of the Organisms.

[From the Department of Bacteriology, College of Agriculture, Edinburgh.]

By **T. Gibson.**

With 2 plates.

Micro-organisms which bring about an ammoniacal decomposition of urea have been investigated frequently since the beginnings of bacteriology, and a large number of ureaclastic bacteria have been described and named. It is clear, however, that many of these organisms have never been adequately characterised, and there is much uncertainty attaching to their classification. The existing information concerning the urea-splitting bacteria of soils is especially indefinite: not only are there difficulties in identifying previously-named organisms, but there is considerable divergence in the findings of different investigators concerning the types which occur in soils. This lack of precise knowledge is probably due to several factors, but it appeared likely that if a considerable number of cultures were isolated by different methods from soils of various types, it would be possible to describe the urea-decomposing microflora of soils more accurately than has hitherto been possible. In this investigation 174 urea-splitting organisms were isolated from soils and studied in pure culture. Nearly two-thirds of the organisms may be classified in the Bacillus Pasteuri group. An account of this group has been published elsewhere (Gibson, 4, 5, 7). Sarcina ureae, which appears to be a common soil organisms, has also been described previously (Gibson, 6). The description and classification of the remaining cultures, some of which appear to be new species, is dealt with in this paper. Part II will contain the results of a quantitative examination of certain soils, a description of experiments which indicate the relative importance of different organisms in the ammonification of urea, and a comparison of the findings of this investigation with those of previous workers.

The artificial Culture of Urea-decomposing Bacteria.

The methods of selective culture used in isolating the organisms referred to in this paper are mainly those which have been described previously (Gibson, 4, 6), but additional information concerning these procedures will be given in Part II.

Without exception, all the urea-decomposing bacteria which have been investigated find optimal conditions for vegetative growth in media containing comparatively high concentrations of peptone and meat extract. Bouillon, agar and gelatin prepared from 1 per cent peptone and 1 per cent meat extract were found to be suitable for general use, and in the following discussion media with this composition are referred to without a qualifying designation. All the organisms, with the exception of a few types which produce a feeble action on urea, either prefer or require alkaline media containing ammonia. These requirements are most easily satisfied by adding

urea prior to sterilisation, provided that the media are used soon after preparation. Agar and gelatin containing 2 per cent urea and sterilised by intermittent steaming, are suitable plating media for the actively urea-clastic bacteria, but lower concentrations of urea allow the less active organisms to grow more profusely. In gelatin for stab cultures 1 per cent urea is sufficient to provide the necessary conditions. In the propagation of stock cultures, where comparatively large amounts of growth are transferred to a new medium, lower concentrations of urea are preferable. A medium suitable for stock cultures of all the organisms is an agar containing 0.25 per cent urea sterilised in the autoclave, but many of the less active organisms may be maintained successfully on ordinary agar with a pH of 7.5. None of the spore-forming bacteria investigated are capable of producing spores freely on substrates which support luxuriant vegetative growth, and in many of the organisms it is difficult to elicit sporulation. An agar which encourages the production of spores by all the types contains 0.5 per cent peptone, 0.5 per cent meat extract and 0.5 per cent NH_4Cl. Its reaction is adjusted to approximately the limiting acidity for the particular strain. Spores are produced more abundantly as a rule at 22° C. than at higher temperatures which permit of more rapid growth.

In this work ability to decompose urea was ascertained by cultivating the organisms in 2 per cent urea bouillon sterilised by steaming, and by titrating 1 ccm quantities of the cultures with standard acid. Organisms were not accepted as being urea decomposers unless they were capable of forming at least 1 mg of ammoniacal nitrogen from urea in each cubic centimetre of culture. It is realised that many micro-organisms which produce some urease are incapable of fulfilling the conditions imposed, but in the past bacteria which do not develop a definite titratable alkalinity in urea media have not been regarded as urea-decomposing types. The standard of urease activity adopted here appears to be a suitable one, for borderline organisms were found infrequently, and it is unlikely that bacteria and fungi which produce a feebler action on urea play an important rôle in the ammonification of this substance in soils.

The Bacillus Pasteuri Group and related Bacteria.

The B. Pasteuri group appears to embrace the most numerous and also the most active urea-splitting bacteria found in soils. In previous publications (4, 5, 7) this group was described as consisting of a series of spore-forming bacilli which require or prefer alkaline media containing ammonia. Several types of the organisms are individually distinctive, but are so completely connected by transitional forms that the group constitutes a relatively homogeneous entity. It is possible, however, to divide the group arbitrarily into three types: (a) B. Pasteuri, which forms spherical or slightly ovoid spores in a terminal or sub-terminal position, and decomposes completely the urea in bouillon containing 2 per cent of this compound within 48 hours; (b) B. Loehnisii, morphologically similar to (a) but acting more slowly upon urea; and (c) B. Freudenreichii, which differs from (b) in producing ovoid to elliptical spores in a central or excentric position. Each of the three species contains strains which differ in the size of the vegetative cell, in the form of colony, in maximum temperature (from under 30° to about 42° C.), in their action on gelatin and nitrates, and in other minor features.

The descriptions already published, which cover 88 strains from soils, include 3 atypical cultures. A few other cultures which appear to be nearly related to bacilli of the Pasteuri group were also isolated at the same time but, on account of certain distinctive features, they were excluded provisionally from the group. The delimitation of the Pasteuri group is somewhat indefinite and it seemed desirable to secure additional cultures of closely-related organisms. Accordingly, further isolations were made recently by means of direct plating, earlier work having indicated that this method yields the greatest proportion of aberrant forms. A series of 25 organisms which, in their general characters, approach closely to the Pasteuri group have now been secured and are reported on here. A proportion of them are undoubtedly variants of bacilli which may be classified in the Pasteuri group. Others might deserve to be named and recognised as species if it were shown, after a sufficient number of strains had been studied, that they constitute well-defined types. The number of strains of each type obtained in this investigation is small and, in the meantime, to consider all the organisms as appendages of the Pasteuri group seems to be the only satisfactory method of classifying them. These organisms appear to be much less widely distributed in soils than those which conform definitely to the Pasteuri group. Only two types (nos. 1 and 6) were obtained from more than two different soils. In the following brief descriptions each type is compared with typical members of the Pasteuri group.

1. Non-motile organisms (4 strains). These bacilli were non-motile when examined immediately after isolation, but in other respects three of them cannot be distinguished from strains of B. Loehnisii and the fourth appears to be an asporogenous B. Pasteuri. It has been shown previously (4) that pure cultures of the Pasteuri group may give rise to non-motile variants. Consequently, in the absence of other distinctive features, lack of motility cannot be accepted as having diagnostic importance.

2. Asporogenous organisms (2 strains). One of these strains is certainly a B. Pasteuri and the other is the non-motile organism referred to above. In the Pasteuri group spore formation is readily lost during artificial cultivation and its absence is of little importance. Even when the conditions mentioned on page 365 are provided, the demonstration of spores in certain strains may be a matter of considerable difficulty.

3. Organisms producing a yellow growth (3 strains). This type differs from B. Loehnisii only in producing a yellow pigment on solid media. On agars of different composition the colour of the growth varies from yellowish-grey to golden-yellow or yellowish-brown, and the sediment at the bottom of slope cultures may become reddish when the liquid evaporates. On potato treated with Na_2CO_3 the growth is bright yellow changing to brownish-yellow. Gelatin is liquefied and nitrates are not reduced. All the three strains gradually lost their chromogenic property in artificial culture, and after some months they produced a grey growth indistinguishable from that of B. Loehnisii. The pigment production may therefore be regarded as an unstable modification.

4. Organisms intermediate between B. Loehnisii and B. sphaericus (2 strains). These bacilli are distinguished from B. Loehnisii by developing easily on ordinary neutral media while neither alkalinity nor the presence of ammonia favour their growth. Most strains of B. Loehnisii are capable of proliferating in ordinary media,

but they have difficulty in initiating growth from small inocula, as on sparsely-seeded plates, unless substrates are alkaline and contain ammonia. It is a matter of opinion whether these intermediate forms should be regarded as a variety of B. Loehnisii or as urea-decomposing strains of B. sphaericus Meyer et Gottheil. The latter alternative is perhaps preferable as it does not obscure the limits of the Pasteuri group in which the conditions of growth are of diagnostic importance.

5. Diastatic organism (1 strain). This is another bacillus which grows as easily on ordinary neutral media as on those which are alkaline and contain ammonia. Unlike bacilli of the Pasteuri group it hydrolyses starch and produces slight acidity (about pH 6.3) in glucose bouillon. In its other cultural characters and in its morphology it cannot be differentiated from strains of B. Freudenreichii which reduce nitrates and liquefy gelatin slowly.

6. Organisms which reduce nitrates to ammonia (5 strains). In each of the species of the Pasteuri group there are strains which reduce nitrates to nitrites and others which produce no action on nitrates. The five strains referred to here differ from B. Loehnisii only in reducing nitrates and nitrites to ammonia. It is doubtful if this character is of sufficient importance to justify their separate classification.

7. Small fluorescent organisms (2 strains). These are small rods which usually measure 0.4—0.5×1—3 μ. They produce a greenish fluorescence in suitable media, and as they have no action on gelatin they appeared at first to be a variety of B. putidum. They differed to some extent from that organism in being peritrichous and Gram-positive, and in their inability to grow in media other than those which are alkaline and contain ammonia. After the organisms had been under observation for a considerable time it was found to be possible to elicit spore formation under the conditions described on page 365. The spores are small spherical or slightly ovoid bodies which are slightly thicker than the vegetative cells, and they are formed terminally by bacilli 3 to 5 μ in length. Spores were never observed in large numbers and, as they do not appear early in the development of a culture, their formation might be easily overlooked. Both strains reduce nitrates and nitrites to ammonia and they appear to be closely related to the last type (No. 6) in which all the organisms are likewise of small size. Their other characters are those of the Pasteuri group. In urease activity one of the strains corresponds to B. Pasteuri and the other to B. Loehnisii.

8. Denitrifying organisms (2 strains). These organisms are larger rods (1 μ or more in thickness) than typical Pasteuri strains, and they frequently produce deeply stainable coccoid and ovoid cells which may reach 3 μ in width. Ovoid spores may be formed, but both cultures became asporogenous soon after isolation. "Rough" variants, which grow as slender rods and filaments, may be separated from typical cultures. Both strains bring about denitrification. In their other characteristics they cannot be distinguished with certainty from types of B. Pasteuri which decompose urea rapidly and fail to liquefy gelatin within two months.

9. Organisms producing large cells (2 strains). When freshly isolated these strains produced large cells varying in form from short rods to coccoids. The majority of the organisms measured 1.5×2 μ and they formed spherical spores 1.3 to 1.7 μ in diameter which produced a swelling

of the mother cell in an excentric position. On solid media the growths were tough and adherent, becoming almost white and wrinkled or mammilated. In liquid media the growth consisted of firm granules without any turbidity. The character of the organism gradually changed in artificial culture. The cells became progressively longer and more slender, spore formation disappeared, the growths on solid media became grey, smooth and easily broken up, and in liquid media the organisms produced an uniform turbidity. The large-celled sporulating form of the organism was distinctly unstable. Frequent pasteurisation of spore-containing growths delayed but seemed unable to prevent the transformation. The final form of the organisms could not be differentiated from asporogenous strains of the Pasteuri group. Physiologically they possessed the features of a B. Loehnisii which liquefies gelatin slowly and fails to reduce nitrate. When cultures of the spore-forming organisms were plated the changes described above took place rapidly. Colonies of the smooth type were produced readily, and when these were cultivated further they gave rise to variants which produced thin irregular colonies with a "ground-glass" appearance. The organisms in the latter types of colony were long, non-sporulating bacilli. These observations suggest that, despite the distinctive character of the original cultures, this type of organism may be a growth form of B. Loehnisii.

10. Glucose-fermenting organism (1 strain). This bacillus produces greater acidity (final pH about 5.6) in glucose bouillon and liquefies gelatin more rapidly than any of the bacilli which definitely belong to the Pasteuri group. In its other characters it is indistinguishable from nitrate-reducing strains of B. Freudenreichii.

11. Organism which forms cylindrical spores (1 strain). This is a small bacillus (usually 0.5×2—$4\ \mu$) which forms slender cylindrical spores little greater in thickness than the vegetative cells. The spores are formed with equal frequency in all positions in the cell. The organism exhibits a feeble action on urea but it requires alkaline media containing ammonia. Growth on artificial media is scanty. Starch is hydrolysed, nitrates are not reduced, and gelatin is not liquefied. This organism shows rather greater distinction from the Pasteuri type than do the other strains described here.

This series of organisms exemplifies the inherent difficulty in making exact definitions for the classification of bacteria. The only conclusions which appear to be warrantable are that types 1 and 2 belong definitely to the Pasteuri group while, if action on nitrates be disregarded, types 6 and 8 may also be included. The distinguishing characters of types 3 and 9 are probably too unstable to permit of their separation from the group. It seems likely that types 4, 5 and 11 exhibit closer affinities to bacteria other than those of the Pasteuri group. But further evidence is required to show the systematic relationships of most of these organisms.

Bacillus lentus n. sp.

Nine strains of this type were studied. They are identical in all essential respects, and they appear to be sufficiently well defined to justify their recognition as a distinct species.

Morphology. (Plate I, figs. 5 and 6). The cells are comparatively short rods and are frequently bent. They generally measure 0.6—0.7×2—$4\ \mu$

in stained preparations. There is little tendency to produce thicker or longer cells, and chains are not formed. Spores, which are generally elliptical, are produced freely. They are variable in size, the largest being about $0.7 \times 1.3\ \mu$. Spores usually develop in an excentric position and they produce a slight swelling of the vegetative cell. Frequently they are formed towards one side of the cell, and their development results in the sporangium becoming asymmetrically distended. Large central spores in fusiform sporangia (one of which is shown in fig. 6) are formed in some cultures.

Motility. The bacilli are motile, but frequently motility is restricted to a few cells in a culture, and it may consist merely of short tumbling movements. The flagella are peritrichous.

Staining. The rods stain uniformly and intensely with aqueous dyes and they retain Gram's stain.

Conditions of growth. Optimum growth occurs in alkaline media containing ammonia, but most strains proliferate easily in ordinary media with a pH of 7—7.5. Growth occurs with difficulty at pH values below 7. Soil extract and the simpler substrates are inferior to media prepared from peptone bouillon. The organisms are aerobes. They decompose urea most rapidly at about 37° C., but the optimum temperature for growth and spore formation is close to 22° C. Cultures on agar remain viable for long periods if protected against evaporation.

Colonies on agar and gelatin. Surface colonies are at first grey but they soon become opaque and almost white. They remain round, flat, glistening, and comparatively small. In some strains they are tough and firmly adherent to the medium. Microscopically they show an entire edge and a featureless interior which soon becomes opaque. Deep colonies are microscopically spherical or discoid.

Agar slope. The growth becomes opaque and grey-white and sometimes tough and adherent, but it remains comparatively thin. It does not spread beyond the inoculated area.

Gelatin stab. The stab growth is thread-like, and a small, non-spreading, grey-white colony develops on the surface. No liquefaction of the gelatin occurs within 4 months in capped tubes.

Glucose agar stab. The stab growth is confined to the upper layers and is thread-like. The surface colony is grey-white and remains small.

Bouillon. A comparatively faint uniform turbidity and later a small amount of sediment are produced. A granular precipitate may be formed on the walls of the tube.

Potato. A visible growth does not appear.

Biochemical characteristics. Milk undergoes no change and no clearing occurs on milk agar. Slight acidity (pH 6—6.3) is slowly developed in glucose bouillon. A similar but still slower change is usually produced in saccharose and lactose media. Starch is hydrolysed. Nitrates are not reduced and indole is not formed. Urea is fermented feebly, 7 days or more being required for the complete decomposition of the urea in bouillon containing 2 per cent of this substance.

Classification. These organisms have not been identified with any previously-described species. Their characters are not greatly different from those of certain strains of B. Freudenreichii, but the cultural characteristics and the action on carbohydrates distinguish the two types, and connecting links have not been discovered. The 9 strains were

obtained from 5 different soils, a result which indicates that they are widely distributed. They constitute a compact group exhibiting uniform characters and little tendency to vary in artificial culture. Accordingly, there appears to be sufficient reason for the recognition of a new species, and the name Bacillus lentus is suggested for this type.

Bacillus fusiformis Meyer et Gottheil.

Two strains of this species were isolated. They are identical with a strain received from Prof. Ford which also produces breakdown of urea. These bacilli exhibit a weak action on urea. They have not been observed to accumulate more than about 2 mg NH_3 nitrogen per ccm in urea bouillon. Adequate descriptions of the species are given by Lehmann and Neumann (12) and by Lawrence and Ford (11), but as the organisms cannot be distinguished morphologically from certain strains of B. Pasteuri and B. Loehnisii the chief differential features will be mentioned. B. fusiformis was found to differ from all organisms of the Pasteuri group by its tendency to spread on the surface of agar, by the production of a brownish, dull, slightly wrinkled growth on the surface of glucose agar stabs, and by its rapid proteolytic action on gelatin and milk. It is further distinguished from all strains of the Pasteuri group, except those which may be regarded as varieties of B. sphaericus, by the ease with which it proliferates on ordinary neutral media and by the rapidity with which cultures exhibit extensive spore formation.

Brunstetter and Magoon (3) mention that they observed dissociation in B. fusiformis, but they give no details of their work, and there appear to be no records concerning the stability of cultures of this organism. Stock cultures of the strains investigated here showed no tendency to change their characters when propagated on agar. It was possible, however, to obtain variants when the organisms were subjected to repeated subculture in milk, or when cultures in other media, which had been incubated for several months at low temperatures, were examined by plating. The variants constitute a continuous series connecting the typical sporing form with a non-sporing type. It will be sufficient, therefore, to refer to the latter. Compared with the sporulating bacillus, the rods are longer and frequently filamentous, and they generally grow in intertwining chains so that growths on solid media are not easily disintegrated. Colonies become ragged in outline but are non-spreading, and they possess a "ground-glass" appearance when examined by transmitted light. The growth on the surface of agar stab cultures does not become wrinkled. The action on gelatin and milk is slower, but this distinction is probably related to the rate of growth. In B. fusiformis urease activity appears to be an unstable character. It disappeared entirely in one of the sporing strains and in its variants. In the case of the other strain this property was lost by two variants which are intermediate in the series, but it was retained by all the extreme asporogenous types which were examined.

Bacillus repens n. sp.

This organism produces a distinctive type of growth on solid media, and it was observed frequently when two soil types were examined by direct plating. It is capable of growing on neutral media which contain neither

urea nor ammonia, and its presence has been noted on plates prepared for the estimation of the total bacterial content of soils when media suitable for that purpose were used. The organism may therefore be of interest for reasons other than its urease activity. Eight strains were investigated in detail. Colonies appearing on plates inoculated with soil, and even the pure cultures, may be mistaken for those of a Proteus or a Zopfii type, and since a tendency to variability was observed in some of the strains, special efforts were made to investigate the production of variants. The description of the organism, as it appeared when freshly isolated, is followed by an account of the variants obtained under laboratory conditions.

Description of Original Cultures.

With the exception of diastatic activity, the 8 strains appear to be identical in all their characters.

Morphology. (Plate I, figs. 1—3). The cells are slender rods with rounded or pointed ends, which usually measure 0.5—0.7×1.5—10 μ in stained preparations. The longer rods (fig. 1) are frequently curved, but the shorter cells (fig. 2) are generally straight. Either of these morphological types may be produced temporarily by a single strain under identical conditions of culture. Short curving chains may be formed, especially in liquid media, and long filaments are not uncommon. Spherical or slightly ovoid spores (fig. 3), which usually measure when mature 0.7—0.9 μ in diameter or 0.7—0.8×0.8—0.9 μ, are formed in a terminal, less commonly in a sub-terminal position. Larger spores are occasionally produced, and many cultures contain a considerable number of very small spores. The smaller spores frequently appear to develop from lateral buds, and may therefore be classified as exospores. Sporulation seldom occurs early in the development of a culture, and usually it is exhibited by only a small proportion of the cells. The heat resistance of the spores is variable. They have been observed to survive exposure at 98—100° C. for 5 minutes but they are generally destroyed in a short time at 90—95° C.

Motility. In young cultures the rods and chains exhibit straight or sinuous movements. The flagella are peritrichous and short. Long rods are furnished with numerous flagella, short rods with few.

Staining. The cells stain uniformly with the usual dyes. They are Gram-negative, but the thickest rods may show a tendency to retain Gram's stain.

Conditions of growth. Alkaline peptone bouillon is a suitable medium. Its value is increased only to a slight extent by the addition of ammonium salts or urea. Growth takes place readily although more slowly at neutrality, but acid media are unsatisfactory. A pH of 6 inhibits multiplication entirely. Soil extract, while capable of supporting growth, is greatly inferior to bouillon. The organism is an aerobe and a psychrophile. It grows well at 20—23° C., but the maximum temperature is under 30° C. Cultures in any medium which permits good growth remain viable for long periods provided desiccation is prevented.

Agar plate. The type of growth is characteristic. Surface colonies are at first thin and grey, and they soon show a tendency to spread. The manner in which they reflect light suggests very fine radial and concentric striation. Microscopically the colonies are finely granular, and bacilli

may be seen spreading outwards on the surface of the agar and forming frequently into rings. Later, when the original colony becomes opaque, the surface of the surrounding medium is covered with almost transparent small daughter "colonies" connected to the parent colony by a very thin surface growth which may be scarcely visible to the naked eye. A microscopic examination at this stage usually shows that the agar has been penetrated by thin, irregular threads which gradually become diffuse. Deep colonies are at first spherical, disc-shaped or ovoid. Later they produce irregular microscopic threads in the medium.

Gelatin plate. Unless a plate is sparsely seeded the medium merely becomes faintly hazy as a result of the formation of microscopic threads in the gelatin and of ring-like growths on its upper and lower surfaces. The gelatin of such plates soon becomes liquefied. When colonies are well separated, each at first appears as a haze which, when examined with a hand-lens, appears to consist of radially-arranged filaments resembling the mycelium of many fungi. Microscopically the colony is found to be growing in the medium and to consist of root-like strands which branch into finer strands and thin threads, any of which may be twisted into spirals. As well-separated colonies become older they frequently possess the naked-eye appearance of a white dust sprinkled on the medium. Microscopically the strands of growth are found to develop swellings of various shapes, the spiral strands become rope-like, and rings of growth appear in the gelatin and on its surface. At this stage the medium soon becomes liquefied. Sometimes when growth occurs slowly and the gelatin has time to concentrate through evaporation, discrete grey colonies may be formed. Microscopically they may be finely granular or they may show a wave-like pattern, and they generally produce some strands and hairs growing into the medium.

Agar slope. The growth on the inoculated area is smooth, glistening and grey, and it becomes opaque. A thin film of growth also spreads over the entire surface of the medium and becomes heaped up at numerous points to form small circular "colonies" which are more transparent than the parent growth. Diffuse growths may penetrate into the agar after incubation for some time.

Glucose agar stab. A thin growth, in which denser areas usually appear, spreads over the surface. In old cultures the surface growth may become thick and brownish-grey. The stab growth is confined to the upper layers in which visible outgrowths may be formed.

Gelatin stab. At first a thin stab growth is produced. This is surrounded by a faint haze in the gelatin, especially near the surface. When examined with a hand-lens the haze is seen to be produced by fine threads. The surface of the gelatin soon becomes densely permeated by threads and begins to liquefy. Small colony-like formations, which are localised swellings of the outgrowths, may appear in any part of the gelatin. A cylindrical liquefaction takes place slowly and a heavy precipitate collects in the liquefied medium. In soft gelatin the outgrowths are thick: in concentrated gelatin their formation may be suppressed.

Bouillon. Growth results in an uniform turbidity and later a greyish slimy sediment which may be dispersed in viscous strings.

Potato. The growth, which frequently fails unless the medium is treated with alkali, is at first yellowish-grey, later brownish-grey and glistening. Occasionally the potato is discoloured.

Biochemical characteristics. Milk is not visibly changed within 2 months. If it is heavily inoculated it may be discoloured or partially curdled after incubation for several months. In bouillon containing glucose or saccharose a slight acidity (pH 6—6.3) is slowly produced. A similar change is brought about in lactose bouillon by one strain only. Starch is hydrolysed by some strains but not by others. Nitrates and nitrites are denitrified. Indole is not formed. Urea is decomposed slowly. In 2 per cent urea bouillon none of the strains ammonify all the urea within 10 days.

Variants of Laboratory Cultures.

Four of the strains have been investigated at different times during a period of four years, and their ability to produce variants which differ from the original cultures has been observed. Most of the results were secured by plating cultures which had been set aside purposely for several months and in some cases for periods longer than a year. Old milk cultures, in which proteolytic changes had started, appeared to be particularly useful. Other methods employed were serial transfer in liquid media and alternate cultivation on potato and in bouillon, the growth from the potato cultures being heated close to the death temperature in the bouillon. Many of the cultures obtained in these ways differed from their parent strains only in the extent to which they formed spores or were capable of spreading on solid media, but three distinctive types of variants were obtained in a stable condition. The latter forms are markedly different from each other, and if their origin were not known it is unlikely that they would be associated in any system of classification. It appears that the distinctions between them are attributable to differences in morphology which can account for profound modifications in the cultural characteristics of these bacteria. The following descriptions are restricted to the three outstanding variants as those of an intermediate character seem to have little significance. The variants are physiologically similar to their parent strains. Differences in rate of action on gelatin, starch and nitrates were observed, but these may be accounted for by the relative rates of growth. Features not referred to in the descriptions are not significantly different from those of the original cultures.

1. Non-motile Variants.

There are no essential morphological distinctions between this type and the motile organisms other than the absence of flagella in the former. The non-motile variants tend to produce spores in greater numbers and of slightly larger size, but organisms which are motile and yet culturally intermediate between the two types may also produce spores prolifically. The cultural characters to be described show that loss of motility results in the organisms becoming incapable of spreading on agar or of boring through agar or gelatin.

Agar plate. Surface colonies are round or slightly irregular; at first grey and showing a granular appearance by transmitted light; later opaque, grey-white and glistening. Microscopically they are at first very finely granular and they show fine wrinkles in various directions, but ultimately they become opaque. Deep colonies are microscopically compact structures with smooth surfaces.

Gelatin plate. Surface colonies are at first small and transparent, later grey and irregularly round. After about 7 days they sink into the gelatin and disintegrate in the liquefying medium. Microscopically they are at first wrinkled and striated in various directions and, as liquefaction occurs, the margins become diffusely woolly in appearance, but there is no formation of threads in the surrounding medium. Deep colonies are microscopically dense and devoid of hair-like outgrowths.

Agar slope. The growth at first possesses a "ground-glass" appearance when seen by transmitted light but soon becomes thick, opaque, grey and glistening. Whitish streaks frequently develop in the old growth. A thinner marginal zone with an undulate edge spreads slowly outwards but does not progress far from the line of inoculation.

Glucose agar stab. The stab growth is thread-like and scanty. The surface growth is grey and glistening in the early stages but later becomes brownish, dull and wrinkled.

Gelatin stab. The stab growth is thread-like but it usually produces a few short, slender, hair-like outgrowths. A small grey colony forms on the surface but soon sinks into a cup-shaped liquefaction which becomes cylindrical.

Bouillon. An uniform turbidity appears and later a grey, granular sediment which is easily shaken up.

Potato. The growth is glistening and grey, changing slowly to a dull brown, and it usually becomes slightly wrinkled. The potato is generally discoloured.

2. Asporogenous motile Variants.

Among the numerous sub-strains which have been investigated all degrees of spore-forming ability were recognised, and one of the original strains was not observed to produce spores until it had been transferred a number of times on artificial media. Sporulation is therefore a characteristic of the organisms which might pass unnoticed, but several variants were obtained which appear to be entirely asporogenous. They were cultivated repeatedly on suitable agars and on potato, and spores were never observed although the cultures were incubated for several weeks. These organisms are identical with the original strains in every way except in their inability to produce spores. Their chief importance is perhaps the difficulty which their classification would entail if they were encountered in nature, for they exhibit a closer resemblance to B. Zopfii Kurth than they do to the better-known spore-forming bacilli.

Cultures which are intermediate between the asporogenous and the spore-forming types may produce large numbers of coccoid bodies which originate in the terminal position or, less frequently, by the development of small buds on the sides of the rods. These bodies are at first Gram-positive, but later they become unstainable. In the latter condition they appear to possess a very fragile membrane, and their heat resistance is low. Cultures containing considerable numbers of them may be destroyed almost immediately at temperatures between 70° and 80° C. Spherical structures of this type have been described as "regenerative bodies" by Löhnis and Smith (15) who found them to be associated with the acquirement of spore formation in asporogenous bacteria. Efforts were made to confirm the findings of these authors that by means of a series of pasteurisations

increasing in severity it is possible to establish the formation of heat-resistant endospores. In two sets of experiments this procedure raised the death temperature from under 5 minutes at 70° C. to over 5 minutes at 95° C., and one of the resulting cultures was a non-motile spore former. In other tests, however, it failed to increase the heat resistance and, as the organisms tended to produce only vegetative bacilli if they were unheated, it would appear that the coccoid bodies formed by the cultures of an intermediate character may be regarded as abortive spores.

3. Asporogenous non-motile Variants.

Organisms of this type were derived from only one of the original cultures. They differ from the latter in the following respects.

Microscopic characteristics. (Fig. 4). The organisms exhibit pleomorphism to a greater degree than the original strains and they show a greater tendency to grow in chains and to form threads. Cultures may contain a variety of morphological types varying from 0.4 to 1 μ in thickness. Thick ovoid cells frequently become numerous as a culture ages. Motility and spore formation are entirely absent.

Agar plate. Surface colonies are at first small and grey with a granular appearance. Later they show an opaque brownish nucleus surrounded by a thinner, ragged margin consisting of growth in the agar. Microscopically the colonies possess at first a folded-hair structure: later, the dense centre is surrounded by a diffuse growth which penetrates only a short distance into the medium. Threads and twisted growths are not formed in the agar. Deep colonies are identical in microscopic structure with the sub-surface growths of colonies which originate on the surface.

Gelatin plate. Surface colonies are round, raised, grey and glistening, and they always remain small. Microscopically they have a smooth circular edge and a finely granular interior, but at an early stage they become entirely opaque. Liquefaction does not occur. Deep colonies appear irregular or ragged to the unaided eye. Microscopically they are at first spherical but they soon produce short twisted strands which invade the gelatin only in the immediate vicinity of the colony nucleus. The strands gradually develop bulbous swellings so that the final microscopic appearance of the colony is that of a circumscribed cluster of small colonies.

Agar slope. The growth is at first grey and moderately dull and it shows a granular appearance. Later it becomes opaque and grows outwards to a very slight extent with a ragged edge. Spreading does not occur.

Gelatin stab. The cultures resemble those of the original strains but the tendency to form outgrowths from the stab is greatly reduced and liquefaction of the medium is exceptionally slow. In tubes protected against evaporation a small quantity of viscous liquid may be found at the surface of the gelatin after about 4 months.

Classification.

No satisfactory description has been found in the literature which applies definitely to the original strains or to any of the stable growth-forms of this organism. The non-sporing motile variant shows considerable similarity to an unnamed urea-decomposing organism described by Santangelo (18). The same variant also resembles, but less closely, the B. vul-

gare var. Zopfii Löhnis (13) which was considered by Löhnis (14) to be identical with Urobac. Miquelii Beijerinck (1), but the latter organisms produce a somewhat different type of spreading growth on solid media. It seems possible that the motile spore-forming strains may have been confused previously with Bact. Zopfii Kurth. Berlioz (2) described the latter as a spore former which liquefied gelatin slowly, and Swellengrebel (19), in a description which indicates little distinction from the strains described here, stated that it forms spores and decomposes urea. Other writers have found that typical B. Zopfii produces no action on either gelatin or urea. In these circumstances it is suggested that the bacilli characterised here should be recognised as a distinct species which may be named Bacillus repens. The evidence collected in this work indicates that the original strains along with their variants constitute a clearly-defined type.

Bacillus carotarum A. Koch.

One strain of this species was observed to produce a slow action on urea, about 1 mg NH_3 nitrogen per ccm. being produced in 2 per cent urea bouillon in 10 days at 22° C. Urease activity appears to be an unstable character of the organism. The strain just mentioned became entirely inactive towards urea soon after isolation, and a second strain obtained from soil was also incapable of producing the decomposition. The strain which originally decomposed urea exhibited considerable variability in its morphological and cultural characters, and the possibility that this phenomenon was associated with the loss of urea-splitting activity was investigated. Four distinctive and comparatively stable growth-forms were separated from the original culture but none of them was capable of attacking urea. Although the results in so far as they concern urease activity were negative, an account of the variants would appear to be of some general interest, for the cultural characteristics of the bacillus have not been described in detail. In the following descriptions, the organism as it was characterised at the time of isolation is referred to as type 1, while the other variants are only briefly described by comparing them with this form. The method of securing the variants consisted chiefly in plating cultures growing on various media and selecting distinctive colonies, the process being repeated if results were obtained.

Type 1.

Morphology. (Plate II, figs. 7, 8 and 9.) In young cultures the cells are variable in size. They measure 0.8—1.7×1.2—5 μ. They vary in shape from thick ovoid cells which become septate without elongating, to more slender rods, and they frequently occur in twisted and felted chains which may be separated only with difficulty. At an early stage the cells become enlarged (up to 2 μ in thickness) and only faintly stainable. Spores (figs. 7 and 9), which vary greatly in size and shape, are produced readily on solid media. They usually measure 0.8—1×1—2 μ, but they may be larger or smaller. Ovoid spores are formed by the shorter cells and elongate spores by the longest rods. The spore produces no swelling of the sporangium and its position in the cell is variable.

Motility is invariably absent.

Staining. In young cultures the cells are uniformly and intensely

stainable and they retain Gram's stain. The large faintly stainable cells are Gram-negative.

Conditions of growth. The organism is capable of growing in simple media such as a mineral solution containing glucose and $(NH_4)_2SO_4$, but growth is more profuse in ordinary media containing peptone. It is an aerobe, and its optimum temperature is close to 37° C., but growth still occurs rapidly at 22° C.

Agar plate. Surface colonies (plate II, fig. 13) are round, markedly raised, often wrinkled, dull, and almost white. The growth is tough. Microscopically, folded chains of bacilli may be seen in the early stages, but the colonies soon become entirely opaque. Deep colonies are microscopically irregular and very dense, sometimes slightly hairy.

Gelatin plate. Surface colonies are round, white and opaque, and they become ragged in outline as the gelatin liquefies. Liquefaction occurs comparatively slowly. Microscopically the colonies are dense and they possess a rough lobed margin with a tangled hair structure. Deep colonies are white and round or irregular. In microscopic structure they resemble surface colonies.

Agar slope. The growth soon becomes thick, tough, opaque and greyish-white. Its surface is usually wrinkled or mammilated and dull. At a later stage grey and white streaks appear in the growth and its surface becomes smooth and glistening. The tough wrinkled growth is produced by intertwining chains of sporing bacilli. The glistening growth consists chiefly of large faintly stainable cells and it breaks up readily in water.

Glucose agar stab. The stab growth is scanty. The surface growth is at first markedly raised, wrinkled and greyish-white; later thick, smooth and greyish; finally brownish.

Gelatin stab. The stab growth produces, especially from the upper part, numerous short hair-like outgrowths. A small greyish-white colony may form on the surface but soon sinks into a funnel- or cup-shaped liquefaction. As the colony sinks it develops a wrinkled surface and a ragged margin.

Bouillon. Granules or flocculent masses of growth appear at the surface from which they readily sink to the bottom of the tube. The medium remains entirely free from turbidity.

Potato. The growth is at first grey-white, dull and sometimes wrinkled. Later it is luxuriant, smooth, and greyish-pink to reddish-brown.

Biochemical characteristics. Milk is acted upon slowly. After 7 to 10 days at 30° to 37° C. clearing begins at the surface and proceeds gradually downwards. Little precipitation of casein occurs. After several weeks the medium becomes brownish. Acids are not formed from glucose, saccharose or lactose, and indole is not produced. Starch is hydrolysed and nitrates are reduced to nitrites.

Type 2.

Morphology. (Plate II, fig. 10.) Young cultures consist chiefly of long rods which occur in chains, but unsegmented threads are not uncommon. The rods measure 0.7—1×2—10 μ, and they may have flat or rounded ends. The chains and threads do not become intertwined to any extent, and the growths may be broken up easily. At an early stage the

cells become thicker and flat at the ends, and they are then stainable only to a slight degree. Spores are not produced.

Agar plate. Surface colonies (plate II, fig. 14) are grey, glistening and irregular, and they possess a crystalline appearance when examined by transmitted light. Microscopically they show the folded-hair structure typical of B. anthracis. Deep colonies are diffuse to the naked eye and microscopically hairy.

Gelatin plate. Surface colonies are grey and irregular in outline. In the early stages they possess a crystalline appearance, but they become opaque as they sink into the gelatin. Microscopically they at first show a wavy-hair structure; later a diffusely tangled-hair appearance with outlying threads. Deep colonies produce outgrowths visible to the naked eye, and when they become large they resemble fungi. Microscopically they consist of radially-arranged branching strands of threads, but they become diffusely hairy when the gelatin begins to liquefy.

Agar slope. The growth is friable, grey and glistening. At first it is thin and granular; later it becomes thicker and spreads with a ragged margin.

Other features. Characters similar to those which distinguish the colonies and slope cultures from those of type 1 also differentiate the cultures in agar and gelatin stabs and on potato. Type 2 produces an "inverted fir tree" growth in gelatin, and its proteolytic action on gelatin and milk is much slower than that of type 1.

Type 3.

Morphology. (Plate II, fig. 11.) The cells in young cultures are rods with rounded ends which occur singly and measure about 0.7—0.8× 1.5—4 μ. Longer and narrower stainable rods and also thicker faintly stainable forms appear as a culture ages. Ovoid to elliptical spores, which usually produce a slight swelling of the cell towards one end, may be formed, but all the cultures examined became asporogenous.

Agar plate. Surface colonies (plate II, fig. 12) are round and glistening and they become thick, grey-white and opaque. Microscopically they are uniformly finely granular, and the margin is entire. Deep colonies are microscopically dense, irregular, and devoid of outgrowths.

Gelatin plate. Surface colonies are grey and round. Microscopically they are uniformly finely granular, and possess a smooth margin which later becomes lobed. Deep colonies are microscopically finely granular and have smooth surfaces.

Agar slope. The growth is grey and glistening and becomes thick and opaque. It may be disintegrated easily in water.

Other features. The growths on all solid media are smooth and friable. Outgrowths are not produced in gelatin stabs, and bouillon cultures become densely turbid in addition to showing a flocculent growth. In other respects this form resembles type 1.

Type 4.

When this variant is cultivated on agar or gelatin the medium becomes reddish-brown. It is otherwise indistinguishable from type 3.

Relationship of the Variants and Classification.

With the exception of type 4, which was secured on only two occasions, all the variants appeared a great many times during the course of the work. The apparent rarity of type 4 may be attributable to the fact that its colonies on agar cannot be distinguished from those of type 3 unless plates are incubated for a sufficiently long period to permit of colour production becoming evident. Cultures of types 2, 3 and 4 were obtained in a stable condition but type 1, the original form of the organism, was relatively unstable, and periodical plating with the selection of typical colonies appeared to be necessary to maintain its purity. All grades of unstable intermediate forms were also observed and, where plating was not resorted to, the majority of cultures tended to remain intermediate between types 1 and 2 or between 1 and 3. The results are in many respects analogous to those obtained by Nungester (17) with B. anthracis. The essential differences between types 1, 2 and 3 appear to be determined chiefly by morphological distinctions. The tough wrinkled growth of type 1 arises through the formation of twisted and firmly intertwined chains of large cells; the anthracoid growth of type 2 is produced by straight or loop-forming chains of long bacilli; while the smooth growth of type 3 and also of type 4 owes its character to the absence of chain formation. The work of Löhnis and Smith (15), Haag (9), and Lehmann and Neumann (12) has indicated that B. megatherium and other large bacilli are especially prone to change into much smaller organisms during artificial cultivation. The case described here is another illustration of this tendency. In the larger spore-forming bacilli the morphological and cultural characters are frequently most distinctive, but the instability of these features imposes a considerable limitation on their value as criteria for identifying the organisms. If the growth-forms of the bacillus described here are considered as variants about a central type, the organism may be identified as Bacillus carotarum A. Koch. The descriptions of that species given by Koch (10), Migula (16), Gottheil (8) and Lehmann and Neumann (12) contain sufficient information upon which to make a diagnosis.

Summary.

A description and classification is given of soil bacteria which bring about the active decomposition of urea, other than those which may be classified in the Bacillus Pasteuri group or as Sarcina ureae. The following are described: (1) A series of strains exhibiting affinities to the Pasteuri group. (2) Bacillus lentus n. sp. (3) Bacillus fusiformis Meyer et Gottheil. (4) Bacillus repens n. sp. (5) Bacillus carotarum Koch. Descriptions are given of the variants produced by laboratory cultures of the three latter species.

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Explanation of Plates.

Figs. 1—11: Preparations from cultures on agar stained with cold aqueous fuchsin. Magnification 1000 ×.

Figs. 1 and 2: Bacillus repens. Two days at 22° C.

Figs. 3 and 4: Bacillus repens. Five days at 22° C.

Figs. 5 and 6: Bacillus lentus. Three days at 22° C.

Figs. 7 and 9: Bacillus carotarum. Two days at 30° C.

Figs. 8, 10 and 11: Bacillus carotarum. One day at 30° C.

Figs. 12 to 14: Colonies of Bacillus carotarum on agar. Two days at 30° C.

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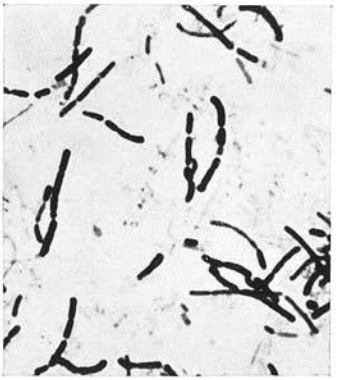

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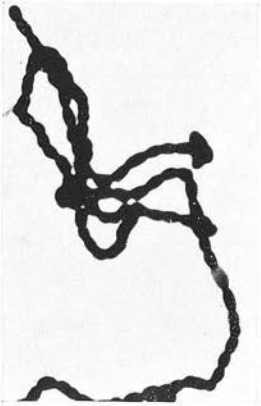

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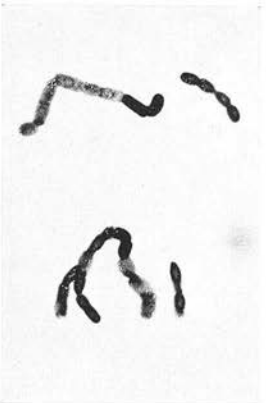

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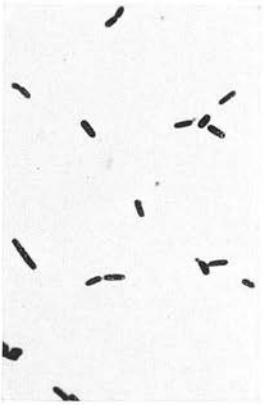

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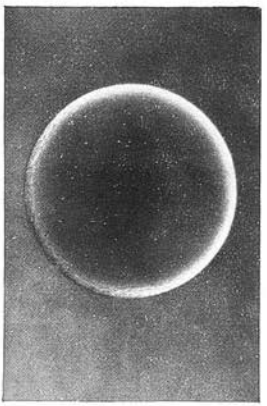

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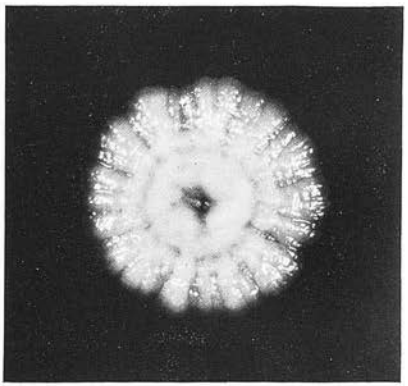

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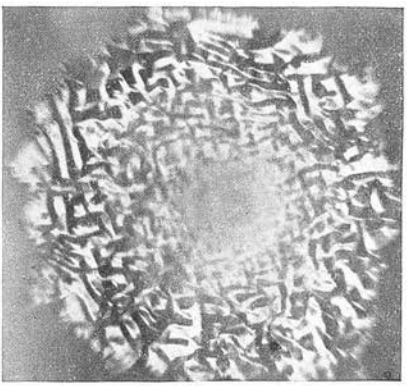

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The Urea-decomposing Microflora of Soils.

II. The Numbers and Types of the Organisms as shown by different Methods.

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By **T. Gibson.**

The introduction by Beijerinck (2) of the principle of selective culture for the isolation of urea-decomposing bacteria from soils and other materials greatly facilitated the investigation of these organisms; and Beijerinck's methods, with minor modifications, have been adopted almost exclusively by later investigators. It appears, however, that these methods, while eminently suitable for the study of the more active urea-splitting bacteria in a soil, yield comparatively little information concerning the less active micro-organisms which may be responsible for the decomposition in the field. Several workers have employed the dilution method to estimate the numbers of ureaclastic bacteria in soils, but no attempt appears to have been made to utilise this technique as a means of securing cultures of those organisms which occur in the greatest numbers.

In this work the urea-decomposing microflora of soils was investigated, not only by employing Beijerinck's methods of enrichment, but also by other procedures yielding information which it is otherwise difficult to secure. The chief objects of the work were the identification and classification of organisms responsible for the decomposition and a study of conditions permitting of their growth. The influence of various factors on the

rapidity of the decomposition in soils has been described previously (Gibson 12); and the results secured in measuring the rate of decomposition in soils with widely different properties have also been published (13). These investigations raised a number of problems concerning the types of organisms which are concerned in the hydrolysis of urea. In particular, it was observed that urea may be rapidly ammonified in soils and peats which appear to be much too acid for the growth of active urea-decomposing bacteria (14, 16). The results of investigating the microflora of several soils are given in this paper, and an effort is made to correlate the findings with those of the previously-described decomposition tests.

The Results of Using Enrichment Cultures.

The use of enrichment cultures, prepared by inoculating urea-containing media with comparatively large amounts of soil, proved to be the only method of isolating the most active urea-decomposing bacteria in a sample. These organisms were found to occur in comparatively small numbers in all the soils examined, and the larger the inoculum the greater is the likelihood of securing their development. Löhnis (31) provides figures which show that the smaller the amount of soil introduced into a medium containing urea the slower and the more incomplete is the resulting ammonification. These results were confirmed in this work, and an investigation of the organisms isolated by different methods showed that the relationship between the intensity of decomposition and the amount of soil used as inoculum was mainly or entirely due to the scarcity of the most active bacteria and to the preponderance of the least active types. For most of the enrichment cultures used in this work 5 g of soil were added to 50 ml of medium, but in some cases double these amounts were employed. The media used were chiefly soil extract (12) and bouillon (1 per cent peptone + 1 per cent meat extract). Urea was added in the proportion of 5 per cent to soil extract and of 10 per cent to bouillon as it was found that these were the approximate concentrations required to suppress bacteria which do not decompose urea. Several other media were tested with one soil but as they did not appear to be selective for particular types of urea-decomposing organisms their use was abandoned. The majority of the cultures were prepared in Erlenmeyer flasks containing a comparatively shallow layer of medium. A few trials were made with tubes containing a deep layer of liquid, but the restriction of aerätion appeared to have no selective effect. The most rapid decomposition was found to occur at about 37° C, but several of the organisms isolated from enrichment cultures incubated at 22° or at 30° were unable to grow at 37°.

From cultures inoculated with six different types of soil 44 organisms were isolated by plating on agar or gelatin containing 2 per cent urea. These organisms do not necessarily represent the types which predominated in each culture at the time of plating, for an effort was made to secure as many distinctive species of bacteria as possible, and colonies were sometimes selected on account of their structure or on account of the morphology of the organisms producing them. The majority of the pure cultures (38 strains) may be classified in the Pasteuri group (15) or in the series of variants of that group described in the preceding paper (16). Typical strains of B. Pasteuri which bring about a rapid decomposition of urea were obtained more frequently than the less active organisms of the group

(B. Loehnisii and B. Freudenreichii). Two acid soils did not yield cultures of B. Pasteuri, but the number of isolations was insufficient to determine whether the organism was entirely absent. Other urea-decomposing bacteria isolated from enrichment cultures are B. repens (1 strain), B. lentus (2 strains), B. fusiformis (2 strains) and B. carotarum (1 strain). These organisms decompose urea very feebly and their isolation is probably to be attributed to their ability to produce colonies which may be distinguished from those of the Pasteuri group rather than to their frequent occurrence in enrichment cultures.

The findings in this work are, in general, confirmatory of Beijerinck's (2) observations concerning the sequence in which different types of bacteria may be isolated from urea-containing media inoculated with soil. The results indicate, however, that the most actively ureaclastic strains of B. Pasteuri are among the first organisms to grow and, provided they occur in the inoculum, it is only their initial small numbers which makes their isolation difficult during the early phases in the development of a culture. The less active organisms may also persist to the extent of being able to produce colonies on the isolation plates up to the stage when the decomposition of urea has reached completion. The strain of B. repens and one of the strains of B. fusiformis were both isolated from completely ammonified cultures. A conclusion which may be drawn from this investigation is that enrichment cultures are necessary for the isolation of B. Pasteuri, but that the less active urea-splitting bacteria which tend to predominate during the early stages in the incubation of these cultures (the "Vorflora" of Beijerinck) may be investigated more effectively by means of the dilution method or by direct plating. The use of these procedures is described in the following sections of this paper.

The Results of Using Dilution Methods.

Dilution methods were employed with the object of providing information concerning the prevalence of urea-splitting bacteria in soils, and also for the purpose of isolating organisms which occur in large numbers but are readily overgrown in the usual enrichment cultures. The media used were 5 per cent urea soil extract and 10 per cent urea bouillon. These solutions were placed in tubes in which approximately 0.5 g of soil had been sterilised previously by steam under pressure, and the complete media were sterilised by intermittent steaming. Soils to be examined were passed through a 3 mm. sieve, dilutions were prepared in multiples of ten, and ten tubes of one or both of the media were inoculated from each of several dilutions. The tubes were placed in a 22° C incubator for four weeks, and all those in which the soil humus had not been dissolved by ammonia were sampled for titration. The most probable number of urea-decomposing bacteria in the soils was estimated by using the tables given by Halvorson and Ziegler (20).

Among the investigators who have employed the dilution method for urea-decomposing bacteria in soils, Rubentschik (38), Fehér (9, 10) and Düggeli (8) observed a seasonal variation in the numbers of the organisms. In this work an experiment was carried out with the purpose of investigating seasonal changes. A plot of garden soil, which received no treatment other than the destruction of weeds by surface hoeing, was sampled at approximately monthly intervals throughout a year. Each test

was made with two composite soil samples which were diluted separately and used for inoculating both the media. The results varied erratically from 28 000 to 2 700 000 per gram in the case of the 10 per cent urea bouillon and from 400 000 to 5 400 000 per gram in that of the soil extract medium, and the figures exhibited no tendency to rise or fall at particular seasons. On several occasions the results obtained from the two series of dilutions were markedly different. The method is obviously not accurate and it does not appear to be capable of demonstrating seasonal fluctuations. One source of error is probably a tendency of the organisms to remain dormant for considerable periods after being introduced into the media. It was observed that the longer tubes were incubated, up to seven weeks, the greater became the number which showed urea decomposition. Some indications were also obtained that different samples of media were not of equal value, and in view of previous observations (12) on the effect of temperature on these organisms, it is likely that the results may be influenced by comparatively slight changes in the temperature of incubation.

Tests were made with other plots of garden soil on which grasses, clover, potatoes and turnips were grown, but none of the crops produced a significant effect on the numbers of urea-decomposing bacteria in the soil.

In a previous paper (12) it was shown that in laboratory tests the reaction of a soil exerts a pronounced effect on the activity of its urea-decomposing microflora. A series of plots of soil, varying in reaction as a result of applying large quantities of calcium compounds to an originally strongly acid soil, were used in that work. It seemed likely that the results obtained were mainly attributable to the scarcity of ureaclastic bacteria in the acid soils, and in order to test this assumption 13 samples from the same series of plots were examined by the dilution method using 5 per cent urea soil extract. The reaction of the samples varied between pH 4.5 and pH 7.6. The results all fell within the limits of 210 000 and 2 400 000 per gram and they exhibited no relation to the reaction of the soils. This finding suggests that the increased activity, brought about by changing the reaction of a soil from the neighbourhood of pH 4.5 to the alkaline side of neutrality, cannot be attributed to an increase in the numbers of its urea-decomposing bacteria. The finding also shows that soils containing approximately the same number of the specific bacteria may exhibit marked differences in activity in solution and soil tests. If the numbers of urea-splitting bacteria bear little relation to their activity in the soil, it may be concluded that the practice of estimating their numbers as a means of characterising a soil from the biological point of view is valueless. The procedure does not appear to be based on any foundation of ascertained facts.

An investigation (13) of mountain and heath soils of exceptionally low agricultural value has shown them to be remarkably active in the decomposition of urea. The results obtained when four types of these soils (in each case 6 samples) were examined by the dilution method, using 5 per cent urea soil extract, are given in Table 1. The soils are all rich in organic matter. With the exception of the peats, they showed values for loss on ignition between 11 and 18 per cent of their oven-dry weights. On the basis of laboratory tests A and B can be described as active types. When urea is added to these soils the rate of decomposition is much more rapid than in any of the fertile cultivated soils which have been examined. Types C and D are inactive in solution tests but in soil tests the decomposition of urea is more

Table 1. Numbers of Urea-decomposing Bacteria in Mountain and Heath Soils.

Soil and herbage types	pH	Numbers per gram
A. Permanently waterlogged	5.0—6.3	10 000—350 000
B. Dry soils. Herbage mainly Festuca ovina and Ulex	4.8—5.2	110 000—920 000
C. Unfertile clay. Herbage mainly Nardus stricta	4.1—4.2	2 000—240 000
D. Peats. Herbage mainly Calluna	3.1—4.3	5 000—170 000

rapid than in the case of arable soils. The results secured by the dilution method again indicate that there is little or no relation between the urea-decomposing activity of a soil and its content of ureaclastic bacteria. It may be pointed out that the statements of Loew (30), that urea-splitting bacteria may be absent from various types of soil, have not been substantiated in this work. The soils which have been examined include the most acid peats, forest soils, and unfertile alkali soils, and the organisms were found in every sample.

The garden soil mentioned in a preceding paragraph was used in tests intended to show the proportion of urea-decomposing bacteria which occur in the form of spores under ordinary soil conditions. Each test was carried out by preparing dilutions in the usual way and also by heating a small quantity of the 1 : 100 dilution at 80° C for 15 minutes, the heated suspension being afterwards diluted to the necessary extent. The results obtained in each test from tubes inoculated with the unheated and pasteurised dilutions are given in Table 2. The figures, while somewhat erratic, indicate that a

Table 2. Proportion of Urea-decomposing Bacteria in Soil occurring as Spores.

Test	Medium	Most probable number per gram	
		Total	Surviving pasteurisation
1	Soil extract + 5 per cent urea	2 300 000	330 000
2	do.	400 000	290 000
3	do.	5 400 000	1 700 000
4	do.	2 800 000	1 700 000
5	Bouillon + 10 per cent urea	2 700 000	620 000
6	do.	1 500 000	660 000

considerable proportion of the organisms occur as spores. It is possible that the proportion of spores is greater than is indicated by these results. In pure culture many of the organisms produce spores or spore-like bodies with a low resistance to heat, and if they behave in a similar manner in soils a proportion of the spores would succumb to pasteurisation at 80° C. It is noteworthy that all the bacteria isolated in this work are spore formers or variants of such organisms.

Twenty-six urea-splitting organisms were isolated in pure culture from tubes inoculated with high dilutions of soils. The majority (21 strains) proved to be types of the Pasteuri group which bring about a slow decomposition of urea. Terminal-sporing bacilli which can be identified as B. Loehnisii were the most numerous. These experiments therefore indi-

cate that B. Loehnisii is the most prevalent urea-decomposing organism in soils. A typical B. Pasteuri was not obtained by this method of isolation. Some of the soils examined contain large numbers of Sarcina ureae, but this organism was never encountered when platings were made from tubes of liquid media inoculated with the highest dilutions which yielded a growth of ureaclastic bacteria. Other types which were isolated by a preliminary use of the dilution method are B. repens, B. lentus and a species of Monilia. The method is, however, unsuitable for cultivating urea-splitting fungi. The Monilia was observed to develop only once in tubes inoculated with high dilutions and on that occasion it was isolated in pure culture.

The Results of Using Direct Plating Methods.

Direct plating methods have proved to be valuable in studying organisms which do not grow readily in liquid media. For the isolation of pure cultures flasks containing 100 ml agar (2 per cent agar, 1 per cent peptone, 1 per cent meat extract, pH 7—7.5) are prepared, and immediately before use the medium is melted, 10 g crystalline urea are added, and after steaming for 10 minutes the agar is cooled and used for plating. Few organisms other than those which decompose urea are capable of growing on the medium. A 5 per cent urea agar is less inhibitive to urea decomposers but it is also less selective. The colonies of bacteria which rapidly produce ammonia from urea are usually surrounded by microscopic crystals in the agar, and an attempt was made to use these media for the counting of colonies on plates poured from soil dilutions. It was found, however, that the crystal formation and the selectivity of the media were not sufficiently definite for this purpose, and comparisons with the results of the dilution method showed that a proportion of urea-decomposing bacteria failed to develop on the plates. Pure cultures are isolated most readily by spreading a suspension of soil on the surface of the agar and incubating the plates at 22 or 30° C.

Direct plating appears to yield a greater variety of organisms than either of the other methods employed and, like the dilution method, it permits of the isolation of the most numerous urea-decomposing bacteria in a soil. For these reasons it was utilised to a greater extent than the other procedures, and 104 cultures were isolated by replating colonies which appeared on plates inoculated with soil. All the strains decompose urea slowly. This finding confirms the results obtained by utilising the dilution method. Both procedures show that the organisms, which on account of their large numbers might be expected to be those chiefly concerned with the decomposition of urea in soils, are not the active ureaclastic bacteria which predominate in the isolations made from enrichment cultures. In the soils examined, the organisms which produced the greatest number of colonies upon the plates were strains of the Pasteuri group together with closely-related bacilli. A typical B. Pasteuri was not encountered and B. Loehnisii was found more frequently than B. Freudenreichii. Direct plating was the only method by which cultures of Sarcina ureae were secured. In certain soils the numbers of this organism were about equal to those of the bacilli just mentioned. The plating method also appears to be the most suitable means of isolating B. lentus, B. repens and urea-splitting fungi, but in many soils these organisms are either absent or numerically unimportant and they do not develop on sparsely-seeded plates. One Ac-

tinomyces, one Mucor and eight strains of a Monilia species, all of which produce a slow action on urea, were isolated by this method. In view of Iwanoff's (24, 25) work, which showed that fungi may form urease only in media containing suitable non-nitrogenous substances, the organisms, and also other fungi isolated from soils, were tested for ureaclastic activity in urea bouillon containing glucose. The Actinomyces and the Monilia were indifferent to the carbohydrate but the Mucor failed to produce an appreciable decomposition except in media containing sugar.

Some of the acid soils referred to in the preceding section were examined by direct plating in an attempt to obtain an explanation of their surprisingly great activity in ammonifying urea. Most of these soils appear to be too acid to support the growth of urea-decomposing bacteria, and their content of such organisms was shown by the dilution method to be comparatively small. A considerable number of fungi have been observed to secrete urease by Miquel (36), Shibata (41), Hagem (19), Kappen (27), Kossowicz (28), Goris and Costy (17), Iwanoff (24, 25) and others, and a search was made for fungi or other aciduric organisms which hydrolyse urea but are incapable of proliferating easily in urea-containing liquid media. Dilutions of the soils were plated on 10 per cent urea agar, but the results were similar to those obtained when urea soil extract was inoculated with the dilutions. The numbers of ureaclastic bacteria were shown to be low, especially in the most acid samples, and the few fungi which appeared on the plates produced little growth. The addition of glucose to the medium was tried but no improvement in the development of fungi was observed. These results were interpreted as indicating that fungi were suppressed by the high concentrations of ammonia which is rapidly formed by bacterial colonies in the agar, and platings of the same soils were made with a more favourable medium in which bacterial growth is restrained. An agar with a pH of 6 containing mineral salts, 1 per cent glucose and 0.25 per cent urea was used. The urea was sterilised by filtration and added to the agar at 45° C. immediately before pouring the plates. Numerous fungi and a smaller number of yeasts and actinomycetes appeared on the plates. Thirty-eight cultures were purified and tested for their ability to decompose urea, but none of the organisms produced more than 0.4 mg of ammoniacal nitrogen per 1 ml in any of several urea-containing media in which they were cultivated. Nevertheless all the organisms were shown to contain some urease by the delicate test of adding the growths to a neutral urea solution containing phenol red and incubating the mixture, along with the necessary controls, at 45° C. These experiments indicate that fungi are unlikely to play an important part in decomposing urea even in the most acid soils. Further evidence confirming this conclusion was obtained by the Cholodny (5) microscopic technique. Slides were placed in soil contained in beakers, 1 per cent urea and sufficient water to bring the soil to its optimum moisture content were added, and at intervals during incubation at 22° C. the slides were withdrawn and examined. A rapid bacterial multiplication took place but the presence of urea retarded the growth of fungi and Actinomyces. Organisms of the two latter groups generally developed to a greater extent in the controls to which no urea was added.

In view of these observations, and also of experiments described pre-

viously (12, 13), it appears to be certain that the great activity of the microflora of acid soils in decomposing urea is attributable to the presence of organic matter in an available form. When comparatively large quantities of urea are added to an acid soil, fungi and other inactive organisms may start the decomposition. The experiments of Brioux (4) and several subsequent workers have demonstrated that soils become more alkaline during the decomposition, and once the process is initiated it is likely to result in localised portions of soil becoming suitable in reaction for the growth of an originally small number of active urea-decomposing bacteria. The existence of the latter in an unfavourable environment may be accounted for by the finding that they are entirely spore-forming types.

Comparison of the Results with those of previous Investigations.

In this discussion reference will be made only to those organisms which have been found to occur in soils. The work described in this and also in a previous (13) paper indicates that if urea-splitting organisms should be introduced into soils from other sources they would be greatly outnumbered by the indigenous bacteria, and further, that the organisms which play the major part in the decomposition are those which belong to the normal soil microflora. Of the various materials which may be applied to soils, farmyard manure would be expected to contain the largest inoculum of urea-hydrolysing bacteria. These studies have shown, however, that frequent and heavy applications of dung can have little if any influence on the numbers and types of ureaclastic organisms in a soil.

In considering the results described here along with those obtained by Beijerinck (2), Löhnis (33), Söhngen (42) and Rubentschik (39), it is apparent that the urea-splitting bacteria most frequently isolated from soils by means of enrichment cultures are organisms closely related to B. Pasteuri. It has now been shown by this investigation that the less active organisms of the Pasteuri group (B. Loehnisii and B. Freudenreichii) may be regarded as being generally the most numerous ureaclastic bacteria in soils.

There is little agreement among different investigators concerning the occurrence in soils of organisms which may be distinguished definitely from bacilli of the Pasteuri group. This position is probably to be explained by variations in methods, by differences in the soils used, and by the comparative rarity of certain organisms. The recently-described spore formers B. lentus and B. repens, which occur numerously in certain soils, were probably isolated in this investigation mainly as a result of utilising new methods. The decomposition of urea by B. fusiformis and B. carotarum does not appear to have been observed by previous workers. These organisms occur in much smaller numbers than other ureaclastic bacteria in the single type of soil in which they were found, and their isolation here is probably to be looked upon as a chance result. Other species of aerobic spore-forming bacilli which are capable of growing easily upon ordinary media were not obtained from any of the soils examined. The isolation of urea-splitting strains of B. mycoides and B. megatherium by Düggeli (7), of B. pumilus by Bierema (3), and of an unidentified bacillus (culture 10 B) by Greaves (18) should therefore be regarded as results which depend upon special methods or do not apply to soils generally.

Sarcina ureae was shown by this work to be numerically one of the chief organisms in certain soils. Other sarcinae were not detected. Some of the common sarcinae found in the air are capable of hydrolysing urea but if these organisms are derived from soils it is likely that they occur there only in small numbers. Micrococci of various types, which differ in colour and in their action on gelatin and milk, are frequently observed to bring about an ammoniacal decomposition in urine, and they have been isolated from soils by Warington (46), Miquel (35), Beijerinck (2), Düggeli (7), Rubentschik (38, 39) and Hucker (22). Micrococci were not encountered, however, in this investigation, and it seems likely that their importance in the decomposition of urea in soils is negligible. A similar conclusion is suggested by Beijerinck's (2) experiments.

No representatives of the Coli-Aerogenes or Proteus groups were observed in this work. Kalantarian (26) and also Düggeli (7) record the isolation of B. vulgare and of coliform organisms, and Löhnis (33) and Kalantarian obtained cultures of a B. vulgare var. Zopfii. Löhnis (34) considered that Beijerinck's (2) Urobac. miquelii and Söhngen's (42) Urobac. Jakschii belong to the Proteus group, but neither of these species has been described in detail. It might be expected that ureaclastic strains of the Proteus and Coli groups would have been observed more frequently if they are widely distributed in soils.

Previously-undescribed fluorescent organisms which, on account of their physiology and their spore-forming ability, are probably variant types of the Pasteuri group were isolated here, but none of the typical members of the Fluorescens group were found in the soils examined. Urea-decomposing strains of B. fluorescens and B. putidum have been isolated from soils previously by Heraeus (21), Warington (46), Liebert (29) and Kalantarian (26). When the methods used by these workers and also by Schellmann (40) are considered, it seems likely that enrichment cultures in urine or other simple media may be necessary for the successful demonstration of ureaclastic fluorescent bacteria in soils. Other urea-splitting soil organisms which were not observed in this investigation are the Mycobacteria studied by Söhngen (43) and Vierling (45), and the organisms resembling Corynebacteria (B. Kirchneri and B. erythrogenes) isolated by Löhnis (32, 33) and Söhngen (42). The ureaclastic activity of these bacteria appears to be of a low order, and it is doubtful if their isolation can be accomplished easily by methods in which tolerance of urea or of ammonia is the principle of selective culture. Christensen's (6) Urobac. Beijerinckii, a non-sporing motile rod which produces a yellow growth, was not encountered in this work. It was claimed that this organism is capable of growing in media containing no organic compound other than urea, but the evidence presented by Christensen cannot be regarded as an adequate proof of his conclusion.

Since Tissier and Martelly's (44) work on meat, obligate anaerobes have been accounted (37, 48) among the urea-decomposing microorganisms of soils. A few attempts were made in this work to isolate these bacilli from anaerobic enrichment cultures but none were successful. Tests of ability to hydrolyse urea were also made with pure cultures of B. Welchii, B. saccharobutyricus, B. tertius, B. sporogenes,

B. tetanomorphus and B. tetani, but in every case the results were negative. Ishikawa (23) also failed to bring about the decomposition with three species of anaerobes. It appears that the rapid ammonification of urea in soils maintained under anaerobic conditions (12, 13) can be attributed to strains of the Pasteuri group (11, 14) or other facultative anaerobes and, possibly under certain circumstances, to the action of urease produced previously by organisms growing in the presence of oxygen.

There are a few records (1, 2, 47) of urea decomposition by Actinomyces, but in the experiments described here only one organism of that genus was isolated which satisfies the test used for the recognition of ureaclastic bacteria. A considerable proportion of the fungi which have been observed to decompose urea (p. 420) are probably types which occur in soils. The evidence adduced in this work indicates, however, that few fungi can be regarded as urea-decomposing organisms in the sense in which that term has been used in bacteriology. It seems probable that actinomycetes and fungi are too sensitive to the action of ammonium carbonate to exhibit urease activity comparable with that of many bacteria.

Summary.

The dilution method, as used for estimating the numbers of urea-decomposing bacteria in soils, gives erratic results and was found to be incapable of demonstrating a seasonal variation. An examination of soils of different character by the dilution method showed that the content of ureaclastic bacteria bears no relation to the rate at which urea is decomposed in the soil. In strongly acid soils, in which the organisms are comparatively scarce, a rapid decomposition of urea appears to be attributable to a high content of available organic matter. The more active bacteria may be incapable of multiplying in acid soils in the absence of urea, and their ability to form spores probably accounts for their occurrence. In the soils examined, the majority of ureaclastic bacteria produce a slow action on urea while the most active organisms occur in small numbers. Direct plating is a suitable method of investigating the most numerous organisms (B. Loehnisii, B. Freudenreichii, B. lentus, B. repens, and Sa. ureae), but enrichment cultures inoculated with comparatively large quantities of soil are necessary for the isolation of the most active type (B. Pasteuri). Fungi and actinomycetes are unable to produce a rapid breakdown of urea. The results of this work are compared with those of previous investigations.

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Factors Influencing the Decomposition of Urea in Soils.

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With 1 figure in the text.

A considerable number of soil microorganisms which are capable of decomposing urea have been studied in pure culture, but little attention has been paid to soil conditions in their influence upon the entire microflora concerned with the ammonification of urea. A short review of the more important work which has been done on the factors affecting the decomposition, with special reference to soil conditions, is given below.

The majority of the urea-decomposing organisms are aerobes (25, 34), but an anaerobic decomposition has been observed (20). Obligate anaerobes isolated from meat (37) and facultative anaerobes from soil (5, 10) have been found to be capable of decomposing urea in the complete absence of oxygen. Surface cultivation of soil has been observed to increase the activity of its urea-decomposing organisms (23). Saturating a soil with water caused the decomposition to be slower than at a lower moisture content (21).

The optimum temperature for the urea-decomposing bacteria in soils has generally been found to be about 30° C., although organisms possessing a lower optimum which are capable of growing below 0° C. have been described (28, 31, 32). An active decomposition of urea in a loam and a slower but distinct decomposition in a sand have been observed at 0° C. (21). A rapid decomposition in a clay soil at 2° C. has been described (7, 8).

It has been found that soil microorganisms are unable to use urea as the sole source of carbon and nitrogen (2, 3), but it has been claimed that certain bacteria in pure culture may do so (6, 36). Unlike most other ammonification processes, the decomposition of urea is facilitated by the presence of available non-nitrogenous substances (1, 2, 12, 13, 14, 15, 16, 17, 34), although a sufficient concentration of the latter permits considerable assimilation of urea of the ammonia produced (3). A great variety of carbon compounds may be used (2, 3, 24, 29, 34). Ordinary meat extract bouillon has generally been found to be the most favourable medium although a more rapid decomposition in soil extract with certain samples of Russian Tschernosem has been observed (18). Soil humus may be used as a source of energy and carbon (6) and may also act in other ways in accelerating the decomposition (35).

Alkaline media or media which rapidly become alkaline as a result of ammonia production have been almost invariably used for the cultivation of the most active urea-decomposing bacteria. The effect of soil reaction on the decomposition has recei-

ved little attention. A more rapid decomposition was observed in a lime-poor sand than in a loam rich in calcium carbonate (38). The addition of lime to an acid soil increased its ability to ammonify urea (26), but the opposite effect has also been recorded (21).

A seasonal variation in the activity of bacteria which decompose urea in soil has been observed (23, 30). Increased activity has been found as a result of partial sterilisation (33) and of drying (27).

Methods.

With a few exceptions which are mentioned later the following methods were used.

Soil Samples. The surface soil to a depth of about 8 cm. was first removed and the sample was taken from the next 5 cm. over an area of about 30 cm. square. Aseptic precautions were found to be unnecessary. The soils were passed through a 3 mm. sieve, thoroughly mixed, and were used for the biological tests in an undried condition as soon as possible after being brought to the laboratory.

Solution Tests. After preliminary trials 2 media were adopted for use: (a) Meat extract peptone bouillon + 10 per cent urea; (b) Soil extract (1 kgm. garden soil and 1 litre tap water autoclaved to 1½ atmospheres, filtered and the filtrate made up to 1 litre) + 0.05 per cent K_2HPO_4 + 5 per cent urea. Weighed amounts of unsterilised urea were placed in sterile 300 cc. Erlenmeyer flasks, and just before inoculation 50 cc. of nutrient solution, which had been sterilised in bulk, were added to each flask by a sterile pipette. A 5 gm. quantity of soil was used as inoculum. After incubation the ammonia in 1 cc. quantities of the cultures was titrated against N/14 H_2SO_4, the contents of the flasks being first properly mixed. A correction for the titre of 1 cc. of fresh medium has been applied to the figures presented. Uninoculated but incubated controls seldom showed an appreciable decomposition in the incubation periods used.

Soil Tests. It was found to be necessary to add a comparatively large quantity of urea to the soils in order to bring out differences in urea-decomposing activity. Amounts of soil corresponding to 50 gm. of oven-dry material were placed in large Petri dishes 11 cm. in diameter, and a quantity of urea solution containing 0.5 gm. of urea was then distributed over the soil from an accurate burette. The standard urea solutions were always freshly prepared from pure, dry urea. The tests were later carried out with half the above quantities of soil and urea in dishes 9 cm. in diameter. With the smaller amount of soil the determination of urea was more easily made. In a few tests the two methods were used simultaneously and the results were not appreciably different. The moisture content of each soil was brought to 65 per cent of its water-holding capacity, distilled water being added if necessary. The covered dishes were placed singly on an incubator shelf where the temperature was 22° to 23° C. If the soils were to be incubated longer than 48 hours, the uncovered dishes were placed in a shallow glass basin with some wet cotton at the bottom and with a glass plate almost completely covering the top, the dishes being separated from each other by strips of wood. With the exception of certain low temperature experiments the dishes were not incubated in piles of more than 3, otherwise temperature differences affected the results.

The Determination of Urea in Soils. Under the experimental conditions selected, the only suitable quantitative method of studying the decomposition in soil was to determine undecomposed urea. Two methods were used for extracting the residual urea from the soils after incubation: (a) The soil in each Petri dish was transferred to a 12 cm. Buchner funnel and was washed with about 450 cc. of distilled water. The water was added in small quantities (about 40 cc.), and each was allowed to pass completely through the soil before the next was poured on. The filtrates were made up to 500 cc. with distilled water. (b) With certain samples leaching in a Buchner funnel was extremely slow. In such cases the soil was transferred to a large flask, distilled water was added to bring the total volume of water to 500 cc., and after thorough shaking some of the liquid was filtered through folded papers. Fifty cc. quantities of the filtrates obtained by either method were placed in 300 cc. conical flasks and sufficient acid was added to render the solutions neutral to methyl red, CO_2 being removed by boiling before finishing the addition of acid. The flasks, provided with stoppers, could be left at this stage for some days. The urea was estimated by an urease method similar to that described by Fox and Geldard (9). A 5 cc. quantity of freshly prepared urease solution was added to each flask, and after the rubber stoppers had been secured by cord the flasks were placed in a 45° C. incubator for

2½ hours. The contents of the flasks were then titrated with N/14 H_2SO_4 using methyl red as indicator, the CO_2 being expelled by boiling. The urease solution was prepared by adding 100 cc. of distilled water to 5 gm. of Soya bean meal. The meal was extracted for 15 minutes with frequent stirring and then sufficient H_2SO_4 was added to bring the mixture to about pH 5.2. The amount of acid required varies with different samples of meal. The acidified extract was allowed to stand for a few minutes, and after filtration through paper in a Buchner funnel a clear enzyme preparation suitable for use was obtained. A further small quantity of acid is needed to bring the enzyme preparation to methyl red neutrality after boiling. The amount required by 5 cc. of the solution was determined separately and the necessary correction applied to the results. A considerable destruction of the enzyme occurs if its solution is acidified to this extent before use. Every standard urea solution and enzyme preparation employed in this work was used in at least one control test for the recovery of urea immediately after its addition to soil, the same quantities of soil and urea being used as in the decomposition tests. In no case was the recovery lower than 97 per cent. The chief source of error in the method is the titration of ammonia in extracts which are coloured by dissolved humus. As the filtrations were frequently slow — for some soils they extended to 13 hours — it appeared probable that a considerable decomposition of urea would occur during the process. It was found, however, that when soil was leached in a Buchner funnel the bulk of the urea was removed by the first wash water, and that the filtrates did not show a measurable decomposition of urea until after one to several days at room temperature.

Soil Reaction. The pH of most of the soils was determined by the quinhydrone electrode (4). The colorimetric drop ratio method (11) was employed for certain alkaline soils.

Loss on Ignition. The values quoted are percentages of the oven-dry weights of the soils.

Experimental Error. Except for a few instances, which are specifically mentioned in the text, all the figures to be presented are averages of 2 or more determinations. In the majority of cases the duplicates gave closely agreeing results. The chief exceptions were soils which produced a slow decomposition and certain samples of acid soil which had received heavy applications of various calcium compounds. The main source of errer in the biological tests was probably, however, a variability of incubator temperatures, and this is not reflected in the degree of agreement between duplicate or parallel tests, as in most instances these were made simultaneously in the same incubator. A measure of the variability of results procured at different times is afforded by the figures obtained in frequent tests with soil from one plot (Section 1).

I. The Decomposition of Urea at different Seasons. Factors affecting Laboratory Tests.

An investigation has been made of the decomposition of urea in a single soil type at different seasons of the year. A plot at the College Garden, Liberton, was used for the purpose. The soil is a sandy loam considerably modified by heavy manuring. The loss on ignition is 15.0 to 15.5 per cent and the pH is 7.0 to 7.6 in different samples of the soil. The plot, which had grown vegetables during the summer, was thoroughly cultivated in the autumn of 1926 and was thereafter left undisturbed except for surface hoeing to suppress weeds. The plot was divided into four sections and periodically four samples were taken, one from each section. All the figures quoted below which relate to this plot are averages of four parallel tests each made with a separate sample.

During 1927 solution tests were made in different months. The average results obtained with soil extract and with bouillon are given in Table 1, along with the temperatures and periods of incubation used. The variations among the four parallels of each test were in all cases much smaller than the fluctuations of the means shown in the table. This proves that the latter fluctuations were produced by factors the intensity of which was distinctly different in successive tests. The results with the bouillon medium are ob-

viously affected by some erratic influence, but those obtained with urea soil extract agree closely at both incubation temperatures and appeared to indicate a seasonal change similar in nature to that found among other microbiological processes in soils.

Table 1. Solution Tests, 1927. Mgm. NH_3 N formed per 1 c.c.

21. Feb.	21. Mar.	18. Apr.	18. May	13. Jun.	30. Aug.	5. Oct.	10. Nov.	12. Dec.
5 per cent urea soil extract at about 21° C. for 72 hours								
4.5	6.6	8.6	10.8	7.6	8.1	7.3	6.0	8.1
5 per cent urea soil extract at about 21° C. for 96 hours								
7.6	9.4	12.5	14.5	10.9	10.8	11.0	9.8	12.2
5 per cent urea soil extract at about 30° C. for 48 hours								
9.6	10.0	13.5	13.9	12.2	9.4	11.9	8.9	14.1
10 per cent urea bouillon at about 21° C. for 48 hours								
1.5	14.3	4.8	4.6	11.8	19.4	6.6	16.7	8.6

During the first part of 1928 solution tests were again made using the soil extract medium in a 30° C. and in a 22° C. incubator. In the former case the ammonia was titrated after 48 hours and in the latter after 72 hours of incubation. The average results are shown in Table 2. As in the previous year the variability of the means was much greater than the variation among the parallel tests of any sampling. The figures indicate that the apparent increase in the urea-decomposing activity of the soil microflora in April, May und June of 1927 was a chance result.

Table 2. Solution Tests, 1928. Mgm. NH_3 N formed per 1 c.c.

Incubation	24. Jan.	20. Feb.	20. Mar.	16. Apr.	18. Apr.	25. Apr.	30. Apr.	7. May	12. May	23. May	28. May	4. June
30° C. 48 hrs. . .	11.9	8.4	12.3	8.6	8.9	9.2	10.3	12.7	9.5	11.7	9.8	13.8
22° C. 72 hrs. . .	7.6	6.5	8.2	7.4	7.6	7.5	8.7	10.6	8.0	9.8	8.8	11.4

An attempt has been made to elucidate the factors which produced the wide variation in the results of solution tests. The moisture content of the soil was determined at each sampling but in neither year could any effect of variations in this factor be detected. The results appeared to be also uncorrelated with rainfall or soil temperature. The time elapsing between taking the samples and inoculating the solutions had likewise no influence. Samples were stored for various periods up to 24 hours at temperatures from 0° to 22° C. without influencing their activity. The possibility of variations in activity occurring within shorter intervals than were used for the tests described above was investigated. A plot was divided into 12-inch squares by stakes and on each day of a week in each of the months August, September, October and December 1928 samples were taken from 4 squares for the inoculation of 4 flasks. The bouillon medium, which had previously given the most erratic results, was employed. So long as the incubation temperature remained constant, the ammonification figures

showed only a slight fluctuation which related to the placing of the flasks in the incubator, and the results in the main were very uniform in the different months. Sampling at various times of the day had also no influence. A short period variation was thus undemonstrable.

Before periodic tests were started the manner in which the results were influenced by volatilisation of ammonia from the flasks was studied. The experiments indicated that when the garden soil was used for inoculating the soil extract medium the results were not greatly affected by this cause until after 48 hours at 30° C. or after 3 to 4 days at 22° C. Figures as high as 20.3 mgm. of ammonia nitrogen in 1 cc., implying the finding of 90 per cent of the original urea nitrogen as ammonia, have been obtained with other soils at 30° C. for 48 hours. At temperatures above 30° C. — from 30° to 37° C. — the ammonification produced by inoculations of the garden soil is very rapid after the 24th hour of incubation and considerable losses of ammonia occur from flasks with cotton plugs. When 10 per cent urea bouillon is inoculated with the same soil the experiments are not appreciably affected by losses of ammonia until after 48 hours in the neighbourhood of 22° C. The final figures obtained in tests with this medium were 38.6 to 41.5, which are equivalent to 89 to 95 per cent of the original urea nitrogen as ammonia.

Table 3. The Effects of Temperature and Volatilisation of Ammonia on the Results of Solution Tests. Mgm. NH_3 N formed per 1 c.c.

Approximate mean temperature			19.5° C.	24° C.	27.5° C.	30° C.	32.5° C.
In soil extract + 5 per cent urea	With cotton plug	At 48 hours	2.4	4.0	7.1	10.2	11.6
		,, 72 ,,	5.9	10.9	11.7	14.1	14.4
		,, 96 ,,	10.1	15.0	14.0	15.7	15.3
	With rubber plug	At 48 hours	2.3	4.1	7.3	10.5	13.6
		,, 72 ,,	5.8	11.2	12.8	15.0	17.5
		,, 96 ,,	10.1	15.9	16.0	17.5	19.3
In bouillon + 10 per cent urea	With cotton plug	At 48 hours	4.8	35.7	—	—	—
		,, 72 ,,	37.3	41.3	—	—	—
		,, 96 ,,	40.1	39.9	—	—	—
	With rubber plug	At 48 hours	4.8	36.1	—	—	—
		,, 72 ,,	38.0	43.2	—	—	—
		,, 96 ,,	43.0	43.4	—	—	—

It was suspected that fluctuations of incubation temperature influenced the results, and during 1928 the incubator regulators were frequently altered while the tests were being conducted in efforts to keep the temperatures uniform. An experiment was arranged to test the effects of different incubation temperatures and of losses of ammonia at each. Flasks containing urea soil extract and urea bouillon were inoculated from one well-mixed sample of the garden soil and were incubated in pairs in various positions in different incubators. One flask of each pair was provided with the usual cotton plug and the other was closed by a rubber stopper. Some of the results are shown in Table 3. The figures demonstrate that incubation temperature has a pronounced influence on the rapidity of the decomposition, especially in bouillon. At 37° C. the decomposition takes place still more rapidly than at the highest temperature given in the table. The final quantities of ammonia found in the flasks closed by rubber stoppers, but opened

each day for sampling, corresponded to 91 to 93.5 per cent of the original urea in the soil extract medium, and to 97 to 100 per cent of that in the bouillon. This proved that only a small amount of ammonia was lost during the sampling of the cultures. It was found that if the incubation was carried out in tubes closed with rubber stoppers, so that little oxygen is available to the bacteria, the decomposition is slower in starting than in cotton-plugged flasks. An experiment was set up to test the possibility of the fermentation being retarded by closing flasks with rubber stoppers. Flasks of urea soil extract inoculated from one sample of soil were incubated at four different temperatures close to 30° C. in sets of 3: (a) with a cotton plug, (b) with a rubber stopper, and (c) with a cotton plug pushed into the neck of the flask which was closed by a 2-holed rubber stopper. A current of air free from ammonia was drawn through the necks of the latter flasks during incubation, and the ammonia which escaped through the cotton plugs was absorbed in standard acid and determined. The results obtained are shown in Table 4. The figures are slightly irregular being procured from single flasks, but they indicate that closing flasks with rubber stoppers has little if any influence on the rate of decomposition. Cotton plugs were, however, used for all of the work in view of the possibility of certain soils being affected by lack of aeration. The use of cotton plugs will tend to diminish differences.

Table 4. Solution Tests. Losses of Ammonia at different Temperatures.

Approximate mean temperature	27.5° C.	29° C.	30.5° C.	32° C.
Mgm. NH_3 N found in 1 cc. of culture:				
In flask with cotton plug	7.1	9.2	10.4	10.9
In flask with rubber plug	7.6	9.0	10.7	12.6
In flask with cotton plug aerated . .	7.1	8.3	10.3	10.6
Mgm. NH_3 N lost per 1 cc. of aerated culture	0.4	0.5	0.6	1.3

Table 5. The Influence of small Differences of Temperature on the Decomposition of Urea in Solutions.

In bouillon + 10 per cent urea incubated 48 hours

Approximate mean temperature (° C.). .	23.0	22.7	22.0	21.7	21.2	20.3	20.0	19.5
Mgm. NH_3 N found in 1 cc.	31.7	29.8	28.2	26.5	24.5	17.3	14.6	4.6

In soil extract + 5 per cent urea incubated 48 hours

Approximate mean temperature (° C.). .	31.5	31.0	30.5	30.0	29.5	28.5	28.0
Mgm. NH_3 N found in 1 cc.	11.5	11.0	10.5	10.1	9.4	8.5	7.9

The results of a more complete experiment on the effect of variations of temperature likely to occur in the incubators used are shown in Table 5. Flasks of urea bouillon and urea soil extract were inoculated from one well-mixed sample of the garden soil and were incubated in sets of 4 in different positions in four incubators. Empty flasks were placed between or around the different sets of test flasks. The estimates of temperature given in the table were made from previous observations using standardised thermometers with insulated bulbs at different positions in the incubators, combined with the use of the same thermometers during the experimental incubation. Each figure given in Table 5 is the average of one set of flasks, the four of which were in close agreement in every case. The two ranges of temperature

given in the table represent approximately the ranges of normal fluctuations at any one point in the capsule-regulated incubators used in this laboratory. This experiment indicates that the chief cause of the fluctuations noticeable in Tables 1 and 2 was variability of incubation temperature. Tests in the bouillon medium are so sensitive to small differences of temperature in the neighbourhood of 21° C. that it is almost valueless for comparative work. At first sight the effect of temperature on the decomposition of urea in this medium appears to be exceptional among biological phenomena, but it is probably associated with the fact that in the soil studied the most numerous and the most active urea-decomposing bacteria occur mainly as spores which require some time to germinate. Even at 30° C., a temperature more nearly approaching the optimum of the organisms, there is little decomposition of urea in bouillon during the first 24 hours, but when the decomposition starts it progresses rapidly.

An examination of the data obtained during 1927 and 1928 made it appear probable that different batches of soil extract had not always equal nutritive value although soil from the same source was regularly used in its preparation. Tests with this medium did not always fluctuate in a similar way to soil tests or to solution tests with bouillon made concurrently in the same incubator. However, when 3 different samples of soil extract were tested simultaneously identical results were obtained.

During a part of 1929 solution tests were carried out with observance of the following precautions. The soil extract was all made at the same time. After being thoroughly mixed it was distributed in flasks which, after sterilisation, were capped to prevent evaporation and were stored until required. Incubation was carried out at approximately 30° C. only, as the tests at this temperature in previous years yielded similar results to those at 21° to 22° C., while the higher the temperature the easier it is to maintain it at a constant level. Although the soil temperature never reaches 30° C. the optimum for the microflora of this soil is still higher. The usual shelves were removed from the incubator and replaced by a single wire-netting shelf on which the flasks were incubated. A board was also placed close to each side to promote circulation of air. The incubator temperature was closely watched and if it were seen to vary the regulator was altered. The average results of these experiments are given in Table 6. The variations have been greatly reduced as compared with the previous series, evidently as a result of the changes in technique. The figures afford no evidence of a seasonal change in the urea-decomposing ability of the microorganisms in the soil investigated.

Table 6. Solution Tests, 1929. Mgm. NH_3 N formed per 1 cc.

16. Feb.	6. Mar.	14. Mar.	23. Mar.	9. Apr.	18. Apr.	1. May	8. May	16. May	27. May
8.8	8.6	9.0	9.4	10.3	10.0	9.8	8.8	9.6	10.2
5. June	12. June	25. June	18. Aug.	26. Aug.	2. Sept.	16. Sept.	25. Sept.	6. Oct.	
9.4	9.7	9.4	10.0	10.4	9.0	9.6	10.5	9.4	

Decomposition tests in the soil itself were made with samples from the same plot on various occasions during 1928 and 1929. The average results are shown in Table 7. A seasonal change in activity was also undemonstrable by this method.

Table 7. Soil Tests, 1928—1929. Urea decomposed per cent in 72 hours.

1928.	27. Feb. 34	20. Mar. 37	16. Apr. 40	23. May 39	13. June 35	14. Aug. 36	6. Nov. 39	8. Dec. 43
1929.	22. Feb. 38	18. Apr. 36	12. June 42	19. Sept. 38	2. Oct. 39			

The experiments made by L ö h n i s (23) showed that the urea-decomposing bacteria of a soil were much more active during summer than at other periods of the year, and that the greater summer activity could not be accounted for by the slightly higher temperature at which the laboratory tests were carried out. The different results obtained in this work may be attributable to differences in the soils used. In particular the soil employed by L ö h n i s was less active, although, when a variety of soils are compared, the type used for this investigation shows only moderate urea-decomposing activity. Without very refined methods it may be impossible to demonstrate seasonal changes in a soil microflora which is capable of producing a rapid decomposition of urea.

II. The Effect of Temperature.

The results quoted in the preceding section show that the activity of urea-decomposing bacteria in soil is markedly affected by temperature. In solution the garden soil exhibited the greatest activity in the neighbourhood of 37° C. Of more importance from the agricultural point of view is the activity of the soil microflora at low temperatures such as prevail during the colder months of the year. Two sets of decomposition experiments in soil have been carried out at low temperatures. Six of the soil samples dealt with in Section V were used for the first. The samples had by this time been stored in the laboratory for two months. A fairly uniform temperature was obtained by sinking covered jugs containing the Petri dishes in the soil at a shaded and sheltered place between two buildings. Advantage was taken of a period of continuous frost at the end of December and beginning of January (1928—29) to carry out the experiment. The Petri dishes containing the soils were first cooled to below 0° C., the urea solution was added to them in a cool room (at about 4° C.) and they were at once placed in previously cooled jugs which were covered and sunk in the soil. The Petri dishes were separated from each other and from the bottoms of the jugs by strips of wood. The temperatures inside the jugs recorded at midday on each day of the experiment were as follows:

Dec.	27.	28.	29.	30.	31.	Jan.	1.	2.	3.	4.
	2.4	2.7	2.4	2.1	2.2		1.4	1.6	1.6	1.6° C.

The mean temperature during the period would be approximately 2° C. Determinations of the undecomposed urea in the soils were made after 5 days and again after 8 days. The urea was extracted from the soils in a cool room where the air temperature was 4° to 5° C. The results (single determinations) are shown in Table 8. Although the soil below the jugs was at a higher temperature during the entire experiment than at the surface, air diffusion round the Petri dishes practically equalised the temperature. The soils were placed in the jugs in the same order as in the table, sample 11 being uppermost for the 5 days period and lowest in the 8 days series.

Table 8. The Decomposition of Urea in Soils at about 2° C.

Sample		11	16	17	20	21	26
Urea decomposed per cent	After 5 days	23	28	30	36	34	24
	After 8 days	35	49	47	55	53	35

Another set of experiments was carried out in an electrically operated refrigerator. Two samples of the garden soil, G 1 and G 2, and two samples of a poor, hillside pasture soil, P 1, (pH = 5.0; loss on ignition = 13.4 per cent) and P 2 (pH = 4.7; loss on ignition = 14.0 per cent) were used for soil tests. The two soil types are respectively representative of moderately active and very active urea-decomposing soils. Immediately after the addition of urea the Petri dishes were placed beside the cooling coils of the refrigerator. After being thoroughly chilled for 3 hours the dishes were placed in a previously cooled wooden box along with beakers containing ice and water. The refrigerator was not opened during the experiment except to remove Petri dishes for urea determinations. Temperatures were read on each occasion. The temperature on the shelf supporting the box varied between 0.8° and 2.2° C., while the shelf immediately above the box was 0.1° to 0.5° C. lower. The mean temperature within the box would be close to 1.5° C. The results of the experiments are shown in Table 9. The figures for the garden samples are based on single determinations. The 7 to 11 days experiments were started later than the remainder and ran partly concurrently with the 17 to 21 days tests. During this period the refrigerator temperature was slightly higher than at the beginning of the experiments. In the case of the pasture soils the duplicate tests were made at different times. Irregularities arising from the placing of the dishes are noticeable.

Table 9. The Decomposition of Urea in Soil at about 1.5° C. Urea decomposed per cent.

Days	1	3	5	7	9	11	13	15	17	19	21
Sample G 1 . . .	4.6	13	21	35	42	56	58	70	80	91	100
Sample G 2 . . .	4.8	13	22	34	41	54	60	72	84	94	100

Days	1		2		3		4	
	a	b	a	b	a	b	a	b
Sample P 1 . . .	19	17	42	39	92	90	100	100
Sample P 2 . . .	21	19	51	47	88	90	100	100

The experiments described above confirm and extend the findings of Littauer (21) and of Couturier and Perraud (7, 8) respecting the decomposition of urea in soils at low temperatures. It may be concluded that the nitrogen of urea has the same availability for plants during periods of cold weather as that of ammonium sulphate.

III. The Effect of Aeration.

A number of experiments have been made to determine the effect of restricting aeration on the decomposition of urea in soils. The soils used were: (a) Six samples of permanently waterlogged soils from hill pastures

(pH = 5.3 to 6.3; loss on ignition = 10.6 to 18.3 per cent). The samples were partially dried prior to the experiments in order to permit the aerobic tests being made at the usual moisture content. (b) Two samples of poor, hill pasture soils which are freely drained and dry during most of the year; P 1 and P 2 described in Section II. (c) Two samples of the garden soil. For the anaerobic tests the Petri dishes containing the soils were incubated in a McIntosh and Fildes jar simultaneously with their aerobic counterparts. In all the incubations the methylene blue indicator in the jar remained uncoloured showing that anaerobiosis had been attained. The average results are shown in Table 10. In every soil but one the amount of anaerobic decomposition represented over 70 per cent of that found aerobically. There is no indication that permanently waterlogged soils are relatively more active than freely aerated soils under anaerobic conditions. Although the conditions in these experiments, where the marshy soils were exposed to considerable aeration before testing, are different from those pertaining to the field, the results obtained indicate that the activity of urea-decomposing microorganisms is not likely to be restricted where there is sufficient oxygen to satisfy the requirements of plant roots.

Table 10. The Effect of restricting Aeration on the Decomposition of Urea in Soils.

Soil Type and Incubation Period	Sample	Urea decomposed per cent		Relative Decomposition Aerobic = 100
		Aerobically	Anaerobically	
Permanently waterlogged; incubated 24 hours	1	65	50	77
	2	60	35	58
	3	40	32	80
	4	46	32	70
	5	89	64	72
	6	60	44	73
Freely aerated hill pasture incubated 24 hours	1	97	92	95
	2	94	75	80
Garden soil; incubated 72 hours	1	36	26	72
	2	35	26	74

IV. The Effect of Adding Organic Matter to Soils.

Previous work on the nutritive requirements of urea-decomposing microorganisms indicated that additions of organic matter to soils would probably assist the ammonification of urea. Soil tests have been carried out to ascertain the effect of adding various forms of organic matter to three soils: (1) Sandy soil (pH = 7.5; loss on ignition = 4.6 per cent); (2) Sandy soil (pH = 5.6; loss on ignition = 5.1 per cent); (3) A sample of the garden soil. The latter was included in the tests as an example of a soil rich in organic matter which produces a comparatively slow decomposition of urea. The additions which were made and the average results obtained are given in Table 11.

The humus was prepared from soil containing a considerable quantity of leaf mould. The soil was extracted with 5 per cent HCl for 3 days with occasional shaking, washed free from acid with distilled water and then extracted with 4 per cent NaOH at room temperature. The supernatant liquid was filtered and the humus in it precipitated by an excess of 10 per cent HCl. The precipitate was washed with distilled water, dried at 45° C.

and one per cent KOH was carefully added to it until the pH was approximately 6 after standing. The liquid was evaporated at 45° C. and the "potassium humate" ground finely in a mortar. The various materials were mixed with the soils immediately before adding the urea solution. The sandy soils were incubated for 24 hours but the garden soil was incubated for 3 days as in the other experiments.

Table 11. The Effect of adding organic Substances on the Disappearance of Urea from Soils.

Addition per 100 gm. of dry Soil	Urea metabolized per cent		
	Sandy Soil (1)	Sandy Soil (2)	Garden Soil
None	14	3.9	41
Xylose 1 gm.	15	13	79
Glucose 1 gm.	16	18	92
„Potassium humate" 1 gm.	44	15	79
Peptone (Bacto) 1 gm.	95	55	100

The figures in Table 11 are somewhat variable, a fact which is perhaps attributable to a different microflora in the three soils. The additions of sugars would involve assimilation of the ammoniacal and possibly also the urea nitrogen, but in certain cases there has been a greater disappearance of urea as a result of their addition than can be accounted for in this way. The addition of the humus preparation increased the rate of decomposition in each soil. The greatest effect has, however, been produced by the peptone. It may be concluded that the soil microflora will decompose urea most rapidly when the carbon and nitrogen requirements of the organisms are completely provided for apart from the urea, but that available carbohydrates will also facilitate the decomposition. These findings corroborate earlier work on the nutritive requirements of the organisms in pure culture.

V. The Effect of Soil Reaction.

Several of the plots laid out by Dr. Williamson and Mr. Comrie of this College to investigate the effects of applying different amounts of lime and other calcium compounds to an acid soil on the College farm of Boghall have been utilised for the study of urea decomposition. The soil of these plots is a brown, heavy loam, in origin alluvium formed from boulder clay. It had become distinctly acid, probably as a result of long periods of waterlogging, and in its untreated condition was unable to grow barley. Samples of the untreated soil had pH values between 4.3 and 5.1. The plots appeared to be suitable for the investigation of urea decomposition in soils differing in reaction without a serious intervention of other factors such as varying amounts of organic matter. The soils showed a loss on ignition of 7.1 to 7.7 per cent. The plots used in this investigation had received the following applications per acre about 3 years previously: (a) None; (b) 3.25 tons calcium carbonate; (c) 12 tons calcium carbonate; (d) 9 tons calcium oxide; (e) 9 tons superphosphate; (f) 5.5 tons mineral phosphate. Samples, each a composite of two, were taken at the end of October when a crop of potatoes was still in the ground. Previous tests had shown that the soil on some of the plots was very variable, and in order to allow of the samples being as thoroughly mixed as possible, the soils were partially dried (to

about 13 per cent of moisture) before being sieved. Decomposition tests were carried out in solution and in the soils themselves. The average results obtained are shown in Table 12, where the samples have been arranged in order of ascending pH. The manurial treatments of the plots from which the samples were derived have not been included in the table because samples from the same plot frequently exhibited large differences in pH, while the results of the tests show a distinct relationship to the reaction of the soils. No specific effect of the large phosphate applications could be detected. With some of the samples the agreement between duplicates was poor and certain of the tests were repeated. The irregularities which were obtained are probably mainly due to the variable nature of the soils. Solution tests were more variable than those in soil. In view of the work of Löhnis (22) this may be attributed to the size of inoculum used. The results of the soil tests made with the most active samples using an incubation of 48 hours were not considered to be perfectly reliable because considerable quantities of the soil humus were dissolved by the ammonia, thus causing the filtrations to be very slow and interfering with the use of the indicator in the titrations. Accordingly, a series of tests was made using samples with a pH between 5.4 and 7.0 and a 24 hours incubation period. Two new samples (17 and 20) were introduced into this experiment to give a better series of pH values.

Table 12. The Decomposition of Urea in Soils differing in Reaction.

No.	pH	Urea decomposed per cent		No.	pH	Urea decomposed per cent		
		In soln. 48 hours	In soil 48 hours			In soln. 48 hours	In soil 24 hours	In soil 48 hours
1	4.3	4.6	8.6	14	5.4	17	22	51
2	4.3	4.7	7.3	15	5.4	33	24	62
3	4.4	5.4	7.9	16	5.5	13	22	64
4	4.7	9.1	8.5	17	6.0	33	25	
5	4.7	8.6	12	18	6.1	40	36	67
6	4.9	10	19	19	6.2	45	34	60
7	4.9	15	28	20	6.6	52	31	
8	4.9	18	34	21	7.0	54	32	59
9	5.1	10	17	22	7.0	78	26	46
10	5.1	16	40	23	7.1	51		57
11	5.1	16	43	24	7.1	44		44
12	5.1	23	52	25	7.8	39		41
13	5.2	14	33	26	7.8	38		38

The results given in Table 12 indicate that the reaction of a soil has a pronounced effect on the activity of its urea decomposing microflora. The relationship is more clearly seen from Fig. 1 where the irregularities have been smoothed out by a pH grouping. In view of previous observations that alkaline media, or media which readily became alkaline through the formation of ammonium carbonate, were most suited to the requirements, of many at least, of the known urea-decomposing bacteria, the rapid decomposition in the acid soils, and also in others mentioned in Sections II and III was quite unexpected. A feature of the results is that solution tests indicate that the optimum soil reaction for the decomposition of urea is about pH 7, while soil tests gave relatively better results with the acid samples and suggested an optimum in the neighbourhood of pH 6. Experiments

with other soils have indicated a similar difference between the results of the two tests. This difference cannot be directly attributed to the production of too alkaline a reaction by the formation of ammonia in the neutral and alkaline soils, for the medium used in the solution tests is poorly buffred and the cultures become more alkaline than the soils. The difference also cannot be explained by a more rapid or complete absorption of ammonia by the colloids in the acid soils. Dr. A. M. Smith kindly tested the absorption of ammonia from $(NH_4)_2CO_3$ solution by several of the soils and found that the cation was as readily absorbed by the alkaline as by the acid samples. A possible explanation is that in soils the decomposition of urea is to a considerable extent brought about by fungi, which, as a group, prefer

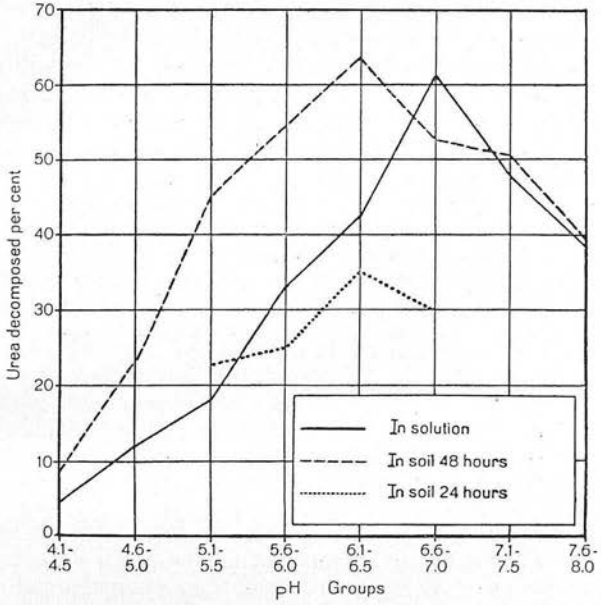

Fig. 1. The influence of soil reaction upon the decomposition of urea.

acid rather than alkaline media. In solution tests the conditions are unsuitable for the development of these organisms and the activity of the bacteria alone is probably measured. Some preliminary experiments have shown that moulds, yeasts, and actinomycetes capable of decomposing urea, although in most cases very slowly, occur in high dilutions from certain acid soils. This problem is being investigated further.

VI. The Effect of Cropping.

The possibility of the urea-decomposing activity of the soil microflora being affected by the growth of crops has been investigated. Plots of the garden soil were sown or planted with (a) grasses (mixed), (b) clover (white), (c) potatoes, and (d) turnips. Several comparative solution tests made during the growing season brought out no distinction between the soil of any of the cropped or uncropped plots. While different crops and their consequent cultivation may have no immediate influence on this decomposition, it has been found that very conclusive distinctions appear between soils main-

tained for long periods under pasture and those which have been uninterruptedly under arable cultivation. In every case examined, urea has decomposed more rapidly in pasture soils than in adjoining arable soils. Details of this work will be published later.

VII. The Effect of Drying Soil.

Some experiments, complementary to those of Rahn (27) on the effect of drying soil, have been carried out. The soils were dried by the method described by Khalil (19) and equal dry weights of the moist and the air-dried soils were used in soil and solution tests. Special precautions were taken in the placing of the flasks and Petri dishes in the incubators so that the tests with the dried and stored portions of each sample should be strictly comparable. The average results obtained in experiments with four samples of the garden soil are given in Table 13. It will be seen that the effect of drying on the rate of decomposition in the soil itself was very slight, and, although the differences are all in the same direction, they are too small to be regarded as significant. The drying has, however, resulted in a more definite increase in the rapidity of ammonification in solution.

Table 13. The Decomposition of Urea in dried and undried Soils. Urea decomposed per cent.

Sample	Fresh Soil		Soil stored 7 Days		Soil dried 7 Days	
	In solution	In soil	In solution	In soil	In solution	In soil
1	44	36	44	36	48	37
2	50	44	49	46	54	49
3	44	35	44	34	48	35
4	49	44	47	42	52	43

These findings lead to the conclusion that, so far as the decomposition of urea is concerned, the chief consequence of drying soil is an activating effect upon the microorganisms themselves rather than upon the soil constituents. When dyring results in the improvement of the soil as a medium for bacterial activity, as has been found for other microbiological processes, the effect is more pronounced in soil tests than in solution tests (19). The majority of the urea-decomposing bacteria in the soil investigated occur as spores, and it is possible that drying stimulates the spores to germinate when subsequently placed in a nutrient liquid, thus increasing the apparent rate of decomposition in solution tests.

Discussion.

The experiments described above, when considered as a whole, show that urea may be decomposed rapidly under very diverse soil conditions. The extraordinary rapidity with which the decomposition may take place in soils at 0° to 2° C. is one of the most significant features of the process. The degree of aeration and the reaction of soils have been shown to influence the change, but the ammonification may occur so readily under the least favourable circumstances with respect to both factors, that neither can be considered as possessing much practical importance. The influence of soil organic matter is almost certainly complicated by the resistance of the ma-

terial. The addition of fresh, available organic matter improves the nutritive conditions for the microorganisms concerned, and pasture soils, which are particularly rich in undecomposed vegetable material, show great urea-decomposing activity. On the other hand, the rich garden soil which was used for much of the work showed only moderate activity. It is probable that in the latter soil the organic matter consists largely of resistant residues which are not readily used by the organisms. In conclusion, it would appear that the availability of urea nitrogen to green plants will, in most agricultural soils, be identical with that of ammonium salts, provided that urea itself is not superior to ammonia as a source of nitrogen. Numerous manuring trials have confirmed this view. The rapid ammonification of urea in many soils shows the danger of nitrogen being lost through the volatilisation of ammonia when urea is applied as a topdressing.

Summary.

1. In laboratory experiments on the activity of the urea-decomposing microflora of soils the results are markedly affected by small variations of incubation temperature when moderately or very active soils are used. — 2. A seasonal variation in the activity of the microorganisms which decompose urea in a garden soil could not be demonstrated either by solution or by soil tests. — 3. Urea is rapidly decomposed in soils at 1° to 2° C. One gram of urea was decomposed by 100 gm. (dry weight) of a pasture soil at about 1.5° C. in less than 4 days. — 4. Urea is decomposed rapidly in soils incubated anaerobically, the amount of anaerobic decomposition usually representing over 70 per cent of that found aerobically. Marsh soils were not more active under anaerobic conditions than freely aerated soils. — 5. Additions of xylose, glucose, humus, and especially peptone to soils increased their ability to decompose urea. — 6. Heavy applications of phosphates to an acid soil produced no demonstrable change in its urea-decomposing activity. — 7. Large applications of lime to an acid soil increased the activity of its urea-decomposing microflora. Samples with a pH of about 7 produced the most active decomposition in solution. Soil tests gave relatively better results with slightly acid samples, the optimum being about pH 6. — 8. Crops of grasses, clover, potatoes and turnips had no detectable influence on the urea-decomposing organisms of the soil during the first season. — 9. Drying a soil resulted in a slightly increased activity in solution tests but the effect in soil tests was doubtful.

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THE DECOMPOSITION OF UREA IN SOILS.

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The decomposition of urea in soils is a microbiological process of some importance in agriculture. In most systems of animal husbandry combined nitrogen is largely returned and added to soil in urea. During the ammonification of calcium cyanamide urea is formed as an intermediate product, and it may also be produced during the decomposition of proteins by micro-organisms (11, 12, 13, 14). In recent years the industrial manufacture of urea from atmospheric nitrogen has increased its importance as a nitrogenous fertiliser.

It has been shown (1, 2, 17, 22, 26, 28) that in various solutions inoculated with soil urea is rapidly converted to ammonium carbonate. The decomposition in soil itself has also been studied quantitatively (4, 5, 6, 7, 15, 16, 18, 19, 23, 27) but only a small number of soils have been examined in this way. In most of the experiments the urea was decomposed very rapidly. Nitrification tests (15, 18, 25) and numerous manuring trials have also indicated that urea is rapidly decomposed in most agricultural soils. It has been stated (20), however, that urea-decomposing bacteria may be absent from various soils, and it appeared to be possible that the slow availability of calcium cyanamide in peaty soils is attributable to the inactivity of these organisms.

In this work the decomposition of urea has been investigated in a wide range of soil types. An attempt has been made to carry out a general survey rather than to demonstrate fine distinctions between particular types of soil.

Methods.

With a few exceptions which will be specified the following methods were used.

Soil samples. The samples were taken from the layer about 3 to 5 inches below the surface over an area of about 1 sq. foot. Aseptic precautions were found to be unnecessary. The soils were passed through a 3 mm. sieve, thoroughly mixed, and used for the biological tests in an undried condition as soon as possible after being brought to the laboratory.

Solution tests. The medium used was soil extract (1 kg. garden soil and 1 litre tap water autoclaved to $1\frac{1}{2}$ atmospheres, filtered and the filtrate made up to 1 litre) + 0·05 per cent. K_2HPO_4 + 5 per cent. urea. The soil extract was all made at the same time. After being thoroughly mixed it was distributed in flasks which, after sterilisation, were capped to prevent evaporation and were stored until required. Weighed amounts of unsterilised urea were placed in sterile 300 c.c. Erlenmeyer flasks, and just before inoculation 50 c.c. of nutrient solution were added to each flask by a sterile pipette. A 5 gm. quantity of fresh soil was used as inoculum, and the flasks were incubated for 48 hours at approximately 30° C. on a shelf of an incubator which maintained an even temperature. After incubation the ammonia in 1 c.c. quantities of the cultures was titrated against $N/14$ H_2SO_4, the contents of the flasks being first properly mixed. A correction for the titre of 1 c.c. of fresh medium has been applied to the figures presented. Uninoculated but incubated controls seldom showed an appreciable decomposition in the incubation period used.

Soil tests. It was found to be necessary to use a very short incubation period and to add a comparatively large quantity of urea to the soils in order to bring out differences in urea-decomposing activity. Amounts of soil corresponding to 25 gm. of oven-dry material were placed in Petri dishes and a quantity of urea solution containing 250 mg. of urea was then distributed over the soil from an accurate burette. The standard urea solutions were always freshly prepared from pure, dry urea. The moisture content of each soil was brought to 65 per cent. of its water-holding capacity, distilled water being added if necessary. The covered dishes were placed singly on an incubator shelf where the temperature was 22° to 23° C. After 24 hours' incubation the undecomposed urea was extracted from the soils and determined by a urease method which has already been described (9). Control experiments on the recovery of added urea have been carried out with a great variety of soils, the same quantities of soil and urea being used as in the decomposition tests. For all the soils examined the percentage of urea recovered never fell below 97 except in the case of peats, and only when the urea was extracted from peat by the method of leaching in a Buchner funnel. The results obtained in a number of tests with different samples of peat, where the peat was leached in a Buchner funnel, can be summarised as follows:

Amount of peat (dry matter) used (gm.)	25	12·5	6·25
Percentage of added urea (0·25 gm.) recovered	90–92	94–97	96–98

The low recovery from 25 gm. (equivalent to about 120 gm. of fresh)

peat is attributable to the sponge-like nature of the material which makes it impossible to replace the absorbed water during filtration. It was concluded that urea undergoes no absorption in any soil.

Soil reaction. The *p*H of most of the soils was determined by the quinhydrone electrode (3). The colorimetric drop ratio method (10) was employed for certain alkaline soils.

Loss on ignition. The values quoted are percentages of the oven-dry weights of the soils.

Experimental error. All the figures to be presented are averages of two determinations, which, in the majority of cases, gave closely agreeing results. The main source of error in the biological tests was probably, however, a variability of incubator temperatures, and this is not reflected in the degree of agreement between duplicate tests, as in most instances these were made simultaneously in the same incubator. A measure of the variability of results secured at different times was obtained by frequent tests with soil from one plot. The standard deviation (8) of the results of 76 solution tests was nearly 7 per cent. of their mean. The results of 56 soil tests with soil from the same plot showed a standard deviation of a little over 10 per cent. of their mean. In the comparisons of arable, pasture and forest soils from each location the tests were made concurrently so that the chief source of error was probably avoided in each comparison.

THE DECOMPOSITION OF UREA IN MOUNTAIN AND HEATH SOILS.

Large areas of Scotland are occupied by natural pastures which, for a variety of reasons, are relatively infertile. Examples of such poor pasture soils occur on the hill section of the College farm at Boghall. The decomposition of urea has been investigated in some of the more distinctive soil types from this source. The average results obtained with six different samples of each are given in Table I along with short notes on the soils. The chief characteristics of the herbage have been included to give an indication of the soil conditions.

The samples of waterlogged soils (Type A) were partially dried before tests were made, and for soil tests they were incubated at 65 per cent. of their water-holding capacity. The figures obtained indicate that the micro-organisms of this soil are capable of decomposing urea rapidly. Soil tests were also made under strictly anaerobic conditions in a McIntosh and Fildes jar. For most of the samples the amount of decomposition which occurred anaerobically represented over 70 per cent. of the aerobic decomposition.

Table I. *The decomposition of urea in mountain and heath soils.*

Soil and herbage types	*p*H	Loss on ignition	Urea decomposed % In solution	Urea decomposed % In soil
Type A				
Soil. Glacial drift, permanently waterlogged by seepage water; generally dark in colour. The low acidity of the soils is associated with the percolation of waters carrying bases from subsurface strata.	6·1	11·7	58	65
	5·3	18·3	44	60
	6·3	15·6	70	40
	6·0	10·6	58	46
	6·0	13·1	30	89
Herbage. Predominantly rushes and sedges; varying proportions of other plants.	6·2	14·0	58	60
Type B				
Soil. Brown, sandy loam, either glacial drift or hill-wash derived from basalts or basic andesites; freely drained; dry during most of the year.	4·7	16·2	32	40
	5·2	14·4	31	42
	4·8	16·8	51	97
	5·0	15·6	55	95
Herbage. Mainly *Festuca ovina*, to a smaller extent *Agrostis vulgaris*, forming a short dense turf with an abundance of fibrous roots. *Ulex* becomes dominant if not destroyed.	4·7	17·3	49	92
	4·9	14·9	27	91
Type C				
Soil. Heavy boulder clay, grey in colour, tending to be wet, especially during winter.	4·5	12·7	3·8	21
	4·2	13·1	1·6	24
Herbage. Almost entirely *Nardus stricta*; frequently some *Vaccinium*.	5·2	15·3	6·9	17
	4·4	13·8	2·4	20
	5·4	13·3	10	27
	5·0	12·9	11	24
Type D				
Soil. Strongly acid peats occurring as thin layers (not more than 8 inches deep) on rocks (rhyolite, acid andesite and trachyte) with no underlying mineral soil. Different proportions of rock fragments produce the variations in loss on ignition.	3·3	70	22	43
	3·3	81	13	23
	3·3	84	8·5	22
	3·2	71	10	36
	3·1	78	2·8	39
	3·3	85	7·6	29
Herbage. Mainly *Calluna*; some *Vaccinium*; little or no grass.				
Peat from the Island of Lewis	4·7	83	29	22

The dry soils (Type B) mainly gave high results, especially in soil tests. The productivity of these soils is low and it is probable that under natural conditions their normal deficiency of moisture renders them unsuitable media for micro-organisms. Without mechanical treatment the soils would not become wet even after prolonged standing in contact with water. For the purposes of the soil tests it was necessary to mix thoroughly each sample with water by means of a spatula before adding the urea solution. The large quantity of readily available nutrient material contained in the broken-up, fresh rootlets in the samples probably also contributes to their urea-splitting activity under laboratory conditions. The results quoted in the table and also others which were

obtained with the same soil type did not indicate any relationship between the urea-decomposing power of the samples and their fertility as judged by the amount and quality of the herbage produced.

The *Nardus*-growing soils (Type C), which are representative of the least profitable grasslands, exhibited the smallest activity, but the tests in the soil itself demonstrated that they contained an active urea-decomposing microflora. After 48 hours' incubation the different samples had ammonified from 34 to 55 per cent. of the added urea.

The samples of peat from Boghall, which exhibited an extreme degree of acidity, produced only a slow decomposition in solution but gave surprisingly high results in the tests where urea was decomposed in the peat itself. In the latter tests the amount of dry peat used was reduced to 12·5 gm. in order to facilitate the determination of urea, but the usual quantity of urea was added. Thus, if the activity of the peat samples is to be compared with that of other soils on an equal dry weight basis the figures obtained in the "soil" tests with peat should be doubled. Peat from Lewis, which was less acid than the Boghall material, showed no greater activity in the decomposition tests.

THE DECOMPOSITION OF UREA IN AGRICULTURAL AND FOREST SOILS.

The urea-decomposing power of a number of soils occurring on different geological formations in the south-east of Scotland has been determined. The samples were taken from locations where cultivated land, permanent pasture and woodland adjoined each other. The three types were usually sampled at points not more than 50 yards apart. In this way the effect of cultivation and cropping of virgin land on the decomposition of urea could be studied. In choosing the locations for sampling an endeavour was made to avoid situations where the land had been allowed to grow timber, or had been converted into permanent pasture because the soil was less fertile than on the adjacent cultivated ground. The arable soils were under rotational cropping. The pasture soils examined had not been cultivated for at least 50 years. In sampling the forest soils the loose litter of undecomposed materials was first removed from the surface and the sample was taken from the next 2 inches, which generally included various proportions of a peaty layer.

The average results of the decomposition tests are shown in Table II, along with short notes on the soil types and the values for pH and loss on ignition of the samples used. As the three soils in each series were probably in all cases originally similar, the differences of loss on ignition between the arable, pasture and forest samples may be taken to represent

differences of organic matter content. Most of the pasture soils produced a very rapid decomposition of the urea. In both solution and soil tests they all showed greater activity than the samples from adjacent arable ground, and especially in soil tests the differences were frequently large. It is probable that the greater activity of pasture as compared with arable soils is exaggerated in laboratory experiments. Old pasture soils always contain an abundance of fibrous roots which are broken up when

Table II. *The decomposition of urea in various soils. Comparison of arable, pasture and forest soils.*

Soil type*, geological formation and locality		Arable				Pasture				Forest			
				Decomposition (%)				Decomposition (%)				Decomposition (%)	
	Series	*p*H	Loss on ignition	In solution	In soil	*p*H	Loss on ignition	In solution	In soil	*p*H	Loss on ignition	In solution	In soil
Blown sand; at margin of cultivation near the coast; from Dirleton, East Lothian	1	7·5	4·6	53	13	6·7	5·3	84	58	4·5	29·9	76	66
	2	5·6	5·1	11	2·3	7·4	6·0	52	31	6·2	11·3	60	20
Heavy clay; alluvium; from Dunmore Park and Grangemouth, Carse of Stirling	3	6·2	9·2	60	17	6·0	16·4	90	100	5·5	15·6	65	78
	4	6·0	9·7	33	7·8	5·8	14·0	90	98	5·8	12·2	69	50
Brown loam; derived from Lower Silurian; from Stobshiel, East Lothian	5	6·0	8·7	54	20	5·9	10·8	75	78	4·4	18·1	56	28
	6	6·3	8·0	58	26	5·8	12·7	77	97	4·2	38·0	26	30
Brown loam; glacial drift derived mainly from carboniferous limestone and calciferous sandstone; from East Saltoun, East Lothian	7	7·1	4·7	35	7·3	7·0	7·6	59	15	4·8	9·8	15	21
	8	7·5	6·5	39	11	5·4	10·4	71	97	4·4	15·1	16	13
Brown loam; glacial drift over trachyte; from Camptoun, East Lothian	9	7·4	7·6	69	12	6·9	9·4	85	88	5·7	9·7	46	18
	10	7·4	6·8	64	6·8	6·1	8·8	76	99	6·2	13·6	84	76

* The descriptions of the soils apply to the samples taken from the arable and pasture ground. The characteristics of the forest soils were in most cases considerably modified by accumulated organic matter.

the soil is sieved, thus providing the micro-organisms with an amount of fresh organic matter which would only become slowly available to them in an undisturbed soil. It has been shown(9) that additions of fresh organic matter to soils enable the micro-organisms to decompose urea more rapidly. Two pasture samples were much less active than the others in both tests: these were the most alkaline samples and they both gave a comparatively low loss on ignition. Some of the forest soils were decidedly more active in one or both of the tests than samples of neighbouring arable soils, but the differences between the two types

were variable and in certain cases were not significant. The woodland samples which showed the least activity gave either a low *p*H value or a low loss on ignition. Some of the forest samples examined approach closely to peat in their characteristics.

THE DECOMPOSITION OF UREA IN ALKALINE SOILS.

Tests were made with a few alkaline soils in order to widen the survey of soil conditions. Short descriptions of the soils used and the results of the decomposition tests are given in Table III.

Table III. *The decomposition of urea in alkaline soils.*

Soil type, geological formation and locality	*p*H	Loss on ignition	Urea decomposed (%) In solution	In soil	
Brown loam containing many fragments of limestone; glacial drift over carboniferous limestone; from East Saltoun, East Lothian	7·5	15·4	82	1 day	13
Clay; under irrigation; alluvium; from Gemmeza, Gharbia, Egypt	8·0	9·6	47	1 day 7 days 14 days	11 37 62
Tenacious, sticky clay; unfertile; alluvium; from Sidi Omar, Edfina, Egypt	8·7	9·9	6·9	1 day 7 days 14 days	0 3·0 5·2
Tenacious, sticky clay; unreclaimed alkali soil; alluvium; from Delengat, Egypt	9·0	6·7	6·4	1 day 7 days 14 days	3·6 18 30

The two most alkaline samples gave low results but in solution tests both produced an active decomposition after the second day of incubation. It is probable that factors other than alkalinity are responsible for reducing biological activity in the sample from Edfina in Egypt.

DISCUSSION.

There has been much controversy concerning the relative merits of soil and solution tests carried out in the laboratory for the purpose of measuring the activities of micro-organisms in soils. Both tests were used in this work as each appeared to be capable of yielding valuable information. There has, however, been no general agreement between the results obtained by the two methods. It will be observed from the tables that the arable soils gave a greater percentage decomposition of urea in solution than in soil tests while the situation is the reverse with all except two of the hill and heath samples. The conditions under which the decomposition is carried out in the soil tests probably approach

most nearly to those in the field; but such tests are also distinctly artificial, especially in the case of marshy or permanently dry soils. It would appear that solution tests measure chiefly the activity of bacteria, while decomposition experiments in soil give information concerning the soil conditions and possibly also the activity of fungi.

The experiments described above were made without regard to the season of the year, a factor which may have modified the results to some extent. A seasonal variation in the activity of the soil micro-organisms which decompose urea has been found by means of solution tests (21, 24), but attempts to demonstrate this phenomenon by employing either solution or soil tests with a garden soil in the neighbourhood of Edinburgh have failed. The results presented in this paper were obtained with samples collected during the periods January to May and August to September.

The chief result of this investigation is that urea is decomposed with extraordinary rapidity in many soils, and no soil of agricultural value has been encountered in which the decomposition took place slowly as measured by the standards of other microbiological processes. A sufficient number of soil types, including extremely acid peat and strongly alkaline soils, have been examined to justify the assumption that urea may be decomposed readily in all soils capable of supporting plant growth. The rate at which urea may be ammonified is probably greater under all soil conditions than that of nitrification. Although urea is easily washed out of soils, the rapidity with which it is converted to ammonia will largely preclude a loss of nitrogen in this way. It is unlikely that inactivity of urea-decomposing micro-organisms is responsible for a slow availability of calcium cyanamide in any soil.

In view of the facility with which micro-organisms decompose urea the soil conditions which favour or retard their action are of minor importance. The number of samples of each soil type examined in this work was small, while the number of types was considerable, and a detailed analysis of the soil factors influencing the decomposition is impossible. A few tentative conclusions can, however, be drawn from the results. The greater activity of pasture soils and many forest soils as compared with those from adjacent cultivated ground is most probably attributable to their greater content of organic matter. The same factor is probably responsible for the superiority in soil tests of most of the hill and heath samples to those of the arable land. The effect of soil reaction, although moderated or magnified by other factors, is discernible among the results. In soil tests the most active samples of

cultivated soil had *p*H values between 6·0 and 6·5, while the most active of the pasture soils were more acid (*p*H 5·4 to 6·1). Activity tended to decrease in the more acid samples and, to a still greater degree, in alkaline soils. In solution tests, however, the acid soils tended to give low results while the alkaline samples were relatively more active than in soil tests.

It might have been expected that the ammonification of urea would take place most rapidly in soils to which farmyard manure or animal urine were frequently added. The experiments described above, however, give little indication of such a relationship. The soils of woodlands which are seldom frequented by animals may be capable of producing a very active decomposition of urea.

SUMMARY.

The decomposition of urea has been investigated in 59 soil samples of extremely varied character. Urea was decomposed readily in all and very rapidly in most of the samples.

In mountain and heath soils the decomposition was generally active, especially in soil tests. Strongly acid peat samples (*p*H 3·1 to 3·3) decomposed from 0·44 to 0·86 per cent. of their own dry weights of urea in 24 hours at 22° to 23° C.

In solution and soil tests samples from permanent pastures produced a more rapid decomposition than those from cultivated land. In soil tests with pasture samples quantities of urea as large as 1 per cent. of the dry weight of soil were converted to ammonia in 24 hours at 22° to 23° C. Fertile arable soils produced a relatively slow decomposition in soil tests.

Samples of forest soils were generally more active than those from cultivated ground.

Strongly alkaline soils showed comparatively little activity in soil tests.

Although urea is not absorbed by soils its rapid conversion to ammonia will generally prevent losses by leaching.

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